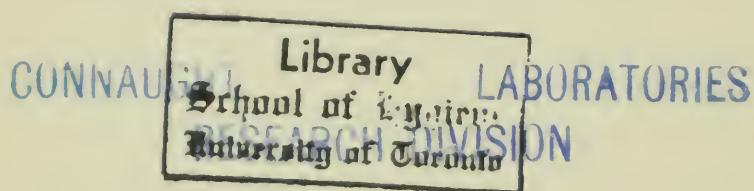




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THE  
JOURNAL OF INFECTIOUS DISEASES



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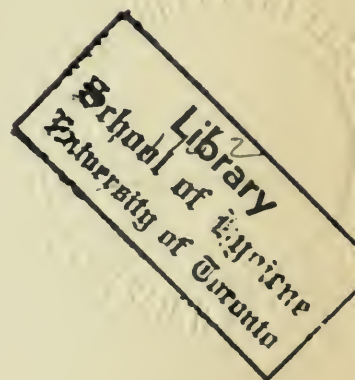
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# A STUDY OF VARIOUS METHODS FOR DETERMINING THE VIRULENCE OF DIPHTHERIA BACILLI \*

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The virulence of a culture of diphtheria bacilli, as accumulated experience has shown, can be determined only by an animal-inoculation test. Differentiation on morphologic characteristics possesses some value, as experience has shown that the majority of cultures of the granular or beaded and barred types of diphtheria bacilli are virulent, whereas the solid types, particularly the short solid varieties, are non-virulent; but between these extremes, including cultures falling into each of these divisions, are many types the virulence of which cannot be foretold with any degree of accuracy according to morphology alone. Likewise, sugar-fermentation tests are at best poor guides for determining the virulence of a culture, altho with dextrose and dextrin virulent bacilli invariably produce acids, while the short solid nonvirulent varieties either fail to produce acids with any sugar or do so in a weak and indefinite manner. The only test of value, therefore, is the animal-inoculation test.

These tests are frequently demanded not only in the laboratories of hospitals for contagious diseases, but in board-of-health and other laboratories as well. This is due to the fact that diphtheria-like bacilli may be found in from 5 to 20% of throats and in from 10 to 20% of noses of healthy persons, so that among a number of persons exposed to diphtheria, some will surely show the presence of these micro-organisms. The proper handling of these contacts depends in many instances on whether or not the bacilli are virulent. Generally speaking, from 30 to 80% of cultures of bacilli from the noses and throats of "carriers" who never have had diphtheria, and never have been intimately exposed to a clinical case of this disease, are nonvirulent; on the other hand, bacilli from the noses and throats of persons who have had diphtheria or who have been intimately exposed to the disease are virulent in from 61 to 100% of cases. In the management of these cases, therefore, particularly from the standpoint of quarantine, the history of the

\* Received for publication December 10, 1915.

patient, the type of bacilli found in the cultures, and, finally, a virulence test, are to be considered.

Likewise, after a patient recovers from an attack of diphtheria, though in the majority of instances the diphtheria bacilli disappear in from 2 to 5 weeks, bacilli may be present on a mucous membrane over prolonged periods of time. In such case, even when cultures are taken in a most careful manner, 2 consecutive negative cultures may be inferior in value to a virulence test, for additional cultures are frequently positive and hence the patient may be released actually carrying bacilli of unknown virulence.

The demand for a virulence test, therefore, is usually based on good reasons. Justification for such a test and the right to pronounce a culture virulent or nonvirulent, with a full realization of the consequences of permitting a person to mingle freely with others in case of the latter, are based mainly on the following:

1. Accumulated experience tends to show that a properly conducted virulence test may be safely accepted as a criterion of the potential pathogenicity of a culture of diphtheria bacilli. The final test is whether a person harboring bacilli nonvirulent for the test animal is capable of infecting other persons; the experience of many investigators, as well as our own, shows that he is not. But as diphtheria so frequently is spread in a varied and confusing manner absolute statements are not justified.

2. Extensive investigations have generally shown that cultures proving nonvirulent in all tests cannot be made virulent by animal passage or other means. Successful results have been reported but not corroborated. On the other hand, attenuated and slight virulence in a strain may be exalted by animal passage or by transfer of the strain to the mucous membrane of another person, and this has an important bearing on the subject of a virulence test, demanding as it does that the test be made as delicate as possible in order that low degrees of virulence may be revealed.

3. While diphtheria bacilli usually retain virulence both on the mucous membranes and under artificial conditions for long periods of time, yet it is probable that in some instances, at least, this property may be lost. We have long held the opinion, however, that nonvirulent bacilli found on the mucous membrane of healthy "carriers" and convalescents are those which are more or less permanently associated with the individual; that they were present on the mucous membranes before

exposure or an attack of the disease and that they are likely to persist for indefinite periods of time after all clinical evidences of infection have disappeared.

The chief requisites for a reliable virulence test may be summarized as follows:

1. The test must be as delicate as possible in order that it may reveal the potential harmfulness of bacilli of low virulence.

2. Any evidence of virulence, however slight, must be regarded as a positive indication of the pathogenicity of a culture under study, for the conditions governing the virulence test are radically different from those controlling infection among men, and it is better to err on the positive side.

3. The test must be specific, conducted with pure cultures. Cultures contaminated with other micro-organisms may show no evidence of virulence because of alteration of the culture medium or the presence of products of the metabolic activity of the contaminating micro-organisms.

4. The test must be conducted with as large a dose of the culture under study as is possible for specific results and the bacilli must have been cultivated under conditions which are most favorable for rapidity of multiplication and the production of toxin.

5. Because colonies of virulent and nonvirulent bacilli may be present side by side on a solid medium, the test should be conducted with a mixture of the bacilli from 2 or more colonies, or 2 or more colonies should be tested separately.

6. The test must be simple and easily interpreted; also economical and possible of completion in as short a time as is consistent with accuracy.

The guinea-pig is most suitable for virulence tests, not only by reason of its size and general adaptability for laboratory work, but because its tissues are peculiarly sensitive to the influence of the diphtheria bacillus and its toxins.

In the Philadelphia hospital for contagious diseases, where the guinea-pig virulence test is frequently used,<sup>1</sup> this study has been conducted during the past 2 years with the purpose of determining what technic best fulfills the requirements of a satisfactory test.

<sup>1</sup> Kolmer, Woody, and Moshage: *Am. Jour. Dis. Child.*, 1916, 11, p. 257. This is a more complete report of 1,054 of these routine tests.

## METHODS OF STUDY

*The Routine Virulence Test.*—The routine test in our laboratory has consisted, briefly, in cultivating pure growths of bacilli in 0.2% dextrose broth, + 0.8 to phenolphthalein, for 72 hours, and subcutaneously injecting guinea-pigs, weighing from 250 to 300 gm., with a dose of the culture corresponding to 0.5% of the body weight, expressed in cubic centimeters, plus sufficient sterile salt solution to make the total volume 4 c.c.<sup>2</sup>

Cultures for use in this routine virulence test are isolated by the "streak" method on plates of Loeffler's serum media and a number of colonies are studied for purity and for types of bacilli. As shown by Arms and Wade,<sup>3</sup> colonies of virulent and nonvirulent bacilli may be present on the same medium; therefore, in an effort to minimize the chances of conducting our test with a non-virulent culture, we inoculate broth with 2 or more different colonies. In all comparative tests for virulence, however, the same cultures are used lest one test be conducted with a particular culture and the comparative test with another.

Unless cultures show a good growth in 24 hours, they are transferred one or more times until they have been educated to grow in a fluid medium. Cultures are grown at 35-37 C. with the tubes slanted in order to expose as large a surface as possible to the oxygen within the tube.

After injection, for at least 4 days, the animals are carefully observed for evidences of local edema and general intoxication. Virulent cultures usually kill the test animal within 3 days, while cultures of low virulence produce some edema, which later subsides, loss in weight and appetite, and other symptoms of toxemia.

When in doubt regarding a result, a second guinea-pig is injected with the culture plus diphtheria antitoxin (1 c.c. of a 500-unit serum). Working with known pure cultures of the bacilli, however, and believing that diphtheria-like bacilli may be safely differentiated from other bacilli by morphologic and staining qualities, supported on postmortem examination by the evidences of true diphtheritic intoxication, such as the characteristic local inflammatory reaction, congestion of the suprarenal glands, pulmonary lesions, etc., we do not consider that every culture requires an antitoxin control. We recommend the control inoculation, however, for laboratories in which these tests are conducted only at irregular intervals.

Since the toxicity and the virulence of diphtheria bacilli are not parallel properties and since toxicity alone represents but one pathogenic factor, all our tests are conducted with unfiltered cultures, so that the combined effect of different pathogenic agents may be studied.

As stated, this subcutaneous method constitutes the routine procedure. We have compared other methods with it in an effort to determine the technic which in our opinion is most suitable and reliable.

*Classification of Diphtheria Bacilli.*—We use the morphologic classification of Westbrook, Wilson, and McDaniel, but for the sake of brevity all the types of bacilli found in the cultures are not recorded. In a culture of granular bacilli A, B, and C types may be found, but if one predominates, the culture is recorded according to that type. The greater proportion of granular bacilli are of these types, and they seem to be of equal virulence. It is frequently difficult to draw sharp lines among the solid bacilli. Length of incubation and particularly the degree of temperature may modify the appearance of bacilli

<sup>2</sup> Jour. Infect. Dis., 1912, 11, 56. Also Weston and Kolmer: Ibid., 1911, 8, p. 295.

<sup>3</sup> Jour. Am. Med. Assn., 1911, 56, p. 809.

to a considerable extent. The cultures for virulence tests were recorded as follows:

- A. Granular bacilli, A, B, C, and D—mostly C.
- B. Barred bacilli, A<sub>1</sub>, B<sub>1</sub>, C<sub>1</sub>, and D<sub>1</sub>—mostly B<sub>1</sub>.
- C. Solid bacilli { Long solid, A<sub>2</sub>, B<sub>2</sub>, and C<sub>2</sub>—mostly C<sub>2</sub>.  
                               { Short solid, D<sub>2</sub> and E<sub>2</sub>—mostly D<sub>2</sub>.

With the subcutaneous method for determining the virulence of diphtheria bacilli as outlined, were compared the following methods:

1. The intracutaneous injection of 72-hour plain-dextrose-broth cultures.
2. The subcutaneous injection of salt-solution suspensions of 24-hour Loeffler cultures.
3. The subcutaneous injection of 72-hour serum-dextrose-broth cultures as compared with the subcutaneous injection of salt-solution suspensions of 24-hour Loeffler cultures.
4. The subcutaneous injection of 72-hour serum-dextrose-broth cultures.
5. The subcutaneous injection of 9-day plain-dextrose-broth cultures.
6. The intraperitoneal injection of 24-hour plain-dextrose-broth cultures.
7. The intraperitoneal injection of 24-hour serum-dextrose-broth cultures.
8. The intraperitoneal injection of 72-hour plain-dextrose-broth cultures.

## RESULTS

*The Comparative Virulence of Diphtheria Bacilli in Subcutaneous and Intracutaneous Injection of 72-Hour Plain-Dextrose-Broth Cultures.*—The results of comparative tests with 37 cultures are shown in Table 1.

In this table the sign + under Subcutaneous Injection indicates that the guinea-pig either died or showed local edema and general toxemia within 4 days after inoculation; the sign + under Intracutaneous Injection indicates the presence of edema and infiltration at the site of injection.

*Method.*—Pure cultures were grown in plain dextrose broth for 72 hours, and 0.1 c.c. of each culture was injected intracutaneously into a guinea-pig, the skin of the abdomen being used after a spot had been bared by pulling out the hairs. At the same time, a second guinea-pig of proper weight received a subcutaneous injection of the same culture. Both animals were observed for a period of 4 days.

Results as follows were observed: Of the 37 cultures, including granular, barred, and solid types of bacilli, 86.5% were positive for virulence with the subcutaneous method, as compared with 64.9% positive with the intracutaneous method. On several occasions there was difficulty in reading the results of the intracutaneous injections, especially in differentiating between a slight degree of edema and the local

nonspecific inflammatory reaction characterized by a small area of infiltration as met with in Römer's intracutaneous test for toxin.

Neisser has suggested an intracutaneous method for testing the virulence of diphtheria bacilli consisting in the intracutaneous injection of 0.1 c.c. of suspensions of bacilli prepared by emulsifying one loopful

TABLE 1

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRACUTANEOUS INJECTIONS OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus	Results	
				Subcutaneous Injection	Intracutaneous Injection
1	Throat.....	14	Barred (B <sub>2</sub> ).....	+	+
2	Nose.....	21	Granular (C).....	+	—
3	Nose.....	19	Granular (C).....	+	+
4	Throat.....	30	Long solid (C <sub>2</sub> ).....	+	+
5	Ear.....	25	Granular (C).....	+	+
6	Throat.....	28	Long solid (C <sub>2</sub> ).....	+	+
7	Ear.....	25	Granular (C).....	+	+
8	Throat.....	20	Long solid (C <sub>2</sub> ).....	+	+
9	Nose.....	18	Granular (C).....	+	—
10	Nose.....	22	Long solid (C <sub>2</sub> ).....	+	±
11	Ear.....	16	Granular (C).....	+	—
12	Throat.....	18	Granular (C).....	+	+
13	Throat.....	16	Long solid (C <sub>2</sub> ).....	+	+
14	Nose.....	18	Granular (C).....	+	+
15	Ear.....	12	Granular (C).....	+	+
16	Throat.....	18	Granular (B).....	+	+
17	Throat.....	17	Granular (C).....	+	+
18	Throat.....	33	Long solid (C <sub>2</sub> ).....	+	—
19	Ear.....	41	Granular (C).....	+	+
20	Ear.....	21	Granular (C).....	+	—
21	Throat.....	39	Granular (C).....	+	—
22	Nose.....	18	Long solid (C <sub>2</sub> ).....	—	—
23	Throat.....	17	Long solid (C <sub>2</sub> ).....	+	+
24	Nose.....	14	Granular (C).....	+	+
25	Nose.....	8	Granular (C).....	+	+
26	Throat.....	22	Long solid (C <sub>2</sub> ).....	+	—
27	Throat.....	17	Granular (C).....	+	+
28	Ear.....	22	Granular (C).....	+	+
29	Throat.....	15	Long solid (C <sub>2</sub> ).....	+	+
30	Nose.....	27	Granular (C).....	+	—
31	Nose.....	15	Short solid (D <sub>2</sub> ).....	—	—
32	Throat.....	33	Long solid (C <sub>2</sub> ).....	+	+
33	Nose.....	26	Granular (C).....	+	+
34	Throat.....	25	Granular (C).....	+	+
35	Nose.....	7	Long solid (C <sub>2</sub> ).....	—	—
36	Throat.....	25	Long solid (C <sub>2</sub> ).....	—	—
37	Throat.....	18	Long solid (C <sub>2</sub> ).....	—	—

The sign + under subcutaneous injection means that the guinea-pig either died or showed local edema and general toxemia within 4 days after inoculation. Under intracutaneous injection, the sign + means the presence of edema and infiltration at the site of injection. The sign — means that there was no evidence of virulence.

of a 24-hour Loeffler slant in 1 c.c., 10 c.c., and 100 c.c. of normal salt solution. As a control, some antitoxin containing 8 units per cubic centimeter is added to an equal volume of the heaviest suspension, and 0.1 c.c. of this mixture injected intracutaneously into the same guinea-

pig. True virulent bacilli produce an area of superficial necrosis in from 48 to 72 hours, whereas the skin at the site of the control injection should remain normal in appearance. One objection to this method is that in using the same animal for both series of injections the antitoxin may influence the sites of the injection of the culture. More recently Zingher and Soletsky,<sup>4</sup> drawing particular attention to this point, modified Neisser's technic to the extent that 1 guinea-pig receives 3 or 4 injections of different cultures (0.1 c.c. of a 24-hour Loeffler's slant suspended in from 25 to 30 c.c. NaCl solution) while a 2nd guinea-pig, serving as the control, receives injections of the same cultures plus 0.5 c.c. of 200-unit antitoxin, injected intracardially just before or intraperitoneally 24 hours previously. One point strongly in favor of the latter method is that of economy, since 2 pigs suffice for 4 or even 6 tests, the method requiring of course that all injections be made at the same time.

As stated, we have experienced some difficulty in reading the reactions with bacilli of low virulence, so that we prefer a method whereby a sufficient amount of culture is injected to elicit well-marked and definite reactions.

*The Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour Plain-Dextrose-Broth Cultures and Subcutaneous Injection of Suspensions of 24-Hour Loeffler Cultures in Sterile Normal Salt Solutions.*—The results of comparative tests with 14 cultures are shown in Tables 2 and 8.

*Method.*—Pure cultures of diphtheria bacilli were grown for 24 hours on slants of Loeffler's blood-serum media in the usual-sized test tubes. After examination of stained smears to insure purity of growth, 10 c.c. of sterile salt solution were used in washing off each culture with the aid of a platinum loop. When a uniform emulsion had been secured, 4 c.c. were injected subcutaneously in the median abdominal line of a guinea-pig weighing from 250 to 300 gm. At the same time the regular subcutaneous test was conducted with a 72-hour plain-dextrose-broth culture of the same micro-organism. All guinea-pigs were observed for a period of 4 days, and if death occurred in any one, it and the control were examined and cultures prepared from the subcutaneous tissues and internal parts.

While the dose was rather large, still in the case of the granular types emulsions of but moderate density had been obtained with 24-hour cultures, and, as previously stated, we believe the test should be conducted in a manner calculated to show low degrees of virulence.

Of 23 cultures, representing granular, barred, long-solid, and short-solid types of diphtheria bacilli, 69.6% were positive for virulence in

<sup>4</sup> Tr. New York Path. Soc., 1915, 15, p. 18. Jour. Infect. Dis., 1915, 17, p. 454.

subcutaneous injection of the emulsion of 24-hour Loeffler cultures, as compared with 65.1% positive in subcutaneous injection of the 72-hour plain-dextrose-broth cultures.

In the case of 10 cultures (Table 2, Nos. 1, 2, 3, 4, 5, 6, and 7; Table 5, Nos. 5, 6, and 9), the results were obtained one or more days

TABLE 2  
COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES AND SALT-SOLUTION SUSPENSIONS OF 24-HOUR LOEFFLER CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Ear.....	39	Granular (C).....
2	Throat.....	21	Granular (C).....
3	Ear.....	3	Granular (C).....
4	Throat.....	15	Granular (C).....
5	Throat.....	14	Long solid (C <sub>2</sub> ).....
6	Throat.....	19	Granular (C).....
7	Throat.....	19	Granular (C).....
8	Throat.....	19	Granular (C).....
9	Throat.....	20	Long solid (C <sub>2</sub> ).....
10	Throat.....	49	Granular (C).....
11	Throat.....	19	Granular (C).....
12	Throat.....	10	Granular (C).....
13	Nose.....	28	Short solid (D <sub>2</sub> ).....
14	Throat.....	16	Granular (C).....

TABLE 3  
COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR SERUM-DEXTROSE-BROTH CULTURES AND SALT-SOLUTION SUSPENSIONS OF 24-HOUR LOEFFLER CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Throat.....	15	Granular (C).....
2	Throat.....	3	Long solid (C <sub>2</sub> ).....
3	Ear.....	18	Granular (C).....
4	Throat.....	16	Granular (C).....
5	Ear.....	28	Granular (C).....
6	Ear.....	30	Long solid (C <sub>2</sub> ).....
7	Nose.....	28	Short solid (D <sub>2</sub> ).....
8	Throat.....	19	Granular (C).....

sooner with emulsions of 24-hour Loeffler cultures than with the routine method employing 72-hour plain-dextrose-broth cultures. In the case of 4 cultures (Table 2, Nos. 6 and 8; Table 5, Nos. 1 and 2), the results with both methods were obtained on the same day. In one case (Table 2, No. 10) the routine method yielded a positive result one day in advance of that from the 24-hour Loeffler culture.

As shown in Table 5, the subcutaneous injection of this dose of an emulsion of 24-hour Loeffler cultures yielded results equal to those obtained with 9-day cultures of the same bacilli in plain dextrose broth. This method is probably the oldest known, having been originally employed by Loeffler, and, as shown in the preceding and following

TABLE 2—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES AND SALT-SOLUTION SUSPENSIONS OF 24-HOUR LOEFFLER CULTURES

24-Hour Salt-Solution Suspension						72-Hour Broth Culture					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
284	4	+	0	0	0	247	1.2	—	—	—	+
262	4	—	+	0	0	268	1.3	—	—	—	+
254	4	+	0	0	0	276	1.4	—	—	—	Toxic
268	4	+	0	0	0	282	1.4	—	+	0	0
294	4	+	0	0	0	304	1.5	—	+	0	0
272	4	—	+	0	0	305	1.5	—	+	0	0
302	4	—	+	0	0	280	1.4	—	—	+	0
278	4	—	+	0	0	266	1.3	—	+	0	0
308	4	—	—	—	—	280	1.4	—	—	—	—
274	4	—	+	0	0	278	1.4	+	0	0	0
280	4	—	—	—	—	276	1.4	—	—	—	—
294	4	—	—	—	—	285	1.4	—	—	—	—
280	4	—	—	—	—	294	1.5	—	—	—	—
288	4	—	+	0	0	298	1.5	—	—	—	Toxic

TABLE 3—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR SERUM-DEXTROSE-BROTH CULTURES AND SALT-SOLUTION SUSPENSIONS OF 24-HOUR LOEFFLER CULTURES

24-Hour Salt-Solution Suspension						72-Hour Serum-Dextrose Broth Culture					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
265	4	—	+	0	0	255	1.3	—	+	0	0
258	4	—	—	—	—	292	1.5	—	—	—	—
270	4	—	+	0	0	268	1.5	—	—	+	0
256	4	+	0	0	0	276	1.4	—	+	0	0
294	4	—	—	+	0	295	1.5	—	—	—	Toxic
300	4	—	—	—	—	280	1.4	—	—	—	—
295	4	—	—	—	—	270	1.4	—	—	—	—
280	4	+	0	0	0	290	1.5	—	—	—	Toxic

tables, it is proved in our experience to be the best, as it saves time spent in performing and reporting on the test and has proved equal or superior to all other methods, in point of delicacy.

*Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour Serum-Dextrose-Broth Cultures and Salt-Solution Suspensions of 24-Hour Loeffler Cultures.*—These comparative tests

TABLE 4

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR  
PLAIN-DEXTROSE-BROTH CULTURES AND SERUM-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Throat.....	19	Granular (C).....
2	Ear.....	32	Granular (C).....
3	Throat.....	21	Long solid (C <sub>2</sub> ).....
4	Nose.....	28	Short solid (D <sub>2</sub> ).....
5	Throat.....	14	Granular (C).....
6	Throat.....	23	Granular (C).....
7	Ear.....	14	Long solid (C <sub>2</sub> ).....
8	Throat.....	34	Barred (B <sub>1</sub> ).....

TABLE 5

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF SALT-  
SOLUTION SUSPENSIONS OF LOEFFLER CULTURES; AND OF 72-HOUR AND  
9-DAY PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus	Salt-Solution Suspension					
				Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
1	Throat	16	Granular (C)	284	4	+	0	0	0
2	Throat	21	Long solid (C <sub>2</sub> )	255	4	+	0	0	0
3	Nose	18	Granular (C)	334	4	+	0	0	0
4	Nose	20	Long solid (C <sub>2</sub> )	380	4	—	—	—	—
5	Nose	26	Barred (B)	304	4	+	0	0	0
6	Throat	19	Granular (C)	276	4	—	+	0	0
7	Nose	30	Short solid (D <sub>2</sub> )	370	4	—	—	—	—
8	Nose	12	Short solid (D <sub>2</sub> )	280	4	—	—	—	—
9	Throat	21	Granular (C)	290	4	—	+	0	0

TABLE 6

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR,  
AND INTRAPERITONEAL INJECTION OF 24-HOUR, PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Throat.....	31	Granular (C).....
2	Nose.....	8	Long solid (C <sub>2</sub> ).....
3	Nose.....	20	Granular (C).....
4	Throat.....	19	Granular (C).....
5	Throat.....	14	Long solid (C <sub>2</sub> ).....
6	Throat.....	18	Granular (C).....
7	Throat.....	19	Granular (C).....
8	Throat.....	19	Granular (C).....
9	Throat.....	25	Granular (C).....
10	Throat.....	19	Granular (C).....
11	Throat.....	22	Long solid (C <sub>2</sub> ).....
12	Throat.....	49	Granular (C).....
13	Throat.....	10	Granular (C).....
14	Nose.....	28	Short solid (D <sub>2</sub> ).....
15	Throat.....	19	Long solid (C <sub>2</sub> ).....

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TABLE 4—Continued

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES AND SERUM-DEXTROSE-BROTH CULTURES

Plain Dextrose Broth				Serum Dextrose Broth			
1 Da.	2 Da.	3 Da.	4 Da.	1 Da.	2 Da.	3 Da.	4 Da.
—	+	0	0	—	+	0	0
+	0	0	0	—	+	0	0
—	—	—	—	—	+	0	0
—	—	—	—	—	—	—	—
—	—	—	+	—	+	0	0
—	—	—	Toxic	—	—	+	0
—	—	—	—	—	—	—	—
—	—	—	Toxic	—	—	—	+

TABLE 5—Continued

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF SALT-SOLUTION SUSPENSIONS OF LOEFFLER CULTURES; AND OF 72-HOUR AND 9-DAY PLAIN-DEXTROSE-BROTH CULTURES

72-Hour Plain-Dextrose-Broth Cultures						9-Day Plain-Dextrose-Broth Cultures					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
275	1.4	+	0	0	0	0	0	0	0	0	0
275	1.4	+	0	0	0	0	0	0	0	0	0
280	1.4	—	—	—	—	300	1.5	—	—	—	Toxic
375	1.4	—	—	—	—	300	1.5	—	—	—	—
280	1.4	—	—	—	+	0	0	0	0	0	0
290	1.5	—	—	—	+	0	0	0	0	0	0
250	1.3	—	—	—	—	250	1.3	—	—	—	—
270	1.4	—	—	—	—	280	1.4	—	—	—	—
285	1.4	—	—	—	Toxic	270	1.4	—	—	—	+

TABLE 6—Continued

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR, AND INTRAPERITONEAL INJECTION OF 24-HOUR, PLAIN-DEXTROSE-BROTH CULTURES

72-Hour Plain-Dextrose-Broth Cultures (Subcutaneously)						14-Hour Plain-Dextrose-Broth Cultures (Intraperitoneally)					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
257	1.3	—	+	0	0	250	1.3	—	—	—	+
280	1.4	—	—	—	—	260	1.3	—	—	—	—
315	1.6	—	—	+	0	255	1.3	—	—	—	—
282	1.4	—	+	0	0	288	1.4	—	+	0	0
300	1.5	—	—	+	0	318	1.6	—	+	0	0
252	1.3	—	+	0	0	285	1.4	+	0	0	0
305	1.5	—	+	0	0	278	1.4	—	+	0	0
280	1.4	—	—	+	0	304	1.5	—	+	0	0
294	1.5	—	+	0	0	304	1.5	—	—	+	0
266	1.3	—	+	0	0	294	1.5	—	+	0	0
280	1.4	—	—	—	—	288	1.4	—	—	—	—
278	1.4	+	0	0	0	290	1.5	—	—	—	Toxic
294	1.4	—	—	—	—	275	1.4	—	—	—	—
300	1.5	—	—	—	—	248	1.2	—	—	—	—
290	1.5	—	+	0	0	260	1.3	—	—	—	—

were conducted with 8 different cultures in the same manner as the preceding tests, except that serum-dextrose broth was employed instead of plain dextrose broth. The results are shown in Table 3.

The addition of serum to dextrose broth (sterile horse serum 1 part, dextrose broth 3 parts) results in richer growths of all types of diphtheria bacilli. As shown in Table 3, the use of the serum broth with the routine method yielded the same percentage (62.5) of positive reactions, as did the emulsions of 24-hour Loeffler cultures in normal salt solution. The latter method, however, showed a slight superiority in that for 4 (Nos. 3, 4, 5, and 8), or 50%, of the cultures the results with this method were known one or more days before the results with the usual method and were also more definite (Nos. 5 and 8).

*The Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour Plain-Dextrose-Broth Cultures and Serum-Dextrose-Broth Cultures.*—The superiority of serum broth over plain broth as a medium for cultivating diphtheria bacilli for virulence tests is further indicated by comparative tests with 8 cultures, the results being shown in Table 4. In all tests the same cultures were used after cultivation in the same incubators for the same length of time.

As shown in Table 4, the serum-dextrose-broth cultures yielded 6 or 75% positive reactions, as compared with 4 or 50% positive reactions with cultures in plain-dextrose broth. The superiority of serum, over plain dextrose broth as a culture medium is also indicated in that earlier (Nos. 5 and 6) or more definite (Nos. 6 and 8) results were obtained with the serum-broth cultures.

*Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of Salt-Solution Suspensions of 24-Hour Loeffler Cultures, and of 72-Hour and 9-Day Plain-Dextrose-Broth Cultures.*—Since the maximal toxin-production by diphtheria bacilli is not usually secured in less than 5 days, we have compared the virulence of 9 cultures, using the regular 72-hour test and 9-day cultures in plain dextrose broth.

*Method.*—Cultures were grown in plain dextrose broth under identical conditions for 72 hours and for 9 days, and injected subcutaneously into guinea-pigs weighing from 250 to 300 gm., according to the general rule. At the same time, for comparison, guinea-pigs were injected with 24-hour Loeffler growths of the same cultures.

The results, shown in Table 5, may be summarized as follows: The 9-day cultures yielded 66.6% positive reactions as compared with 45.5% positive reactions with the 72-hour cultures. This superiority of 9-day cultures was to be expected, but no superiority over the sub-

cutaneous injection of 24-hour Loeffler cultures was shown. Furthermore, the longer time required to conduct a virulence test with 9-day cultures counts against the value of the method for routine work, altho this drawback would be of less significance if greater delicacy were obtained.

*The Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour, and Intraperitoneal Injection of 24-Hour, Plain-Dextrose-Broth Cultures.*—Since absorption from the peritoneal cavity is more rapid than from the subcutaneous tissues, we have sought to determine whether this route of inoculation would render a virulence test more delicate while at the same time shortening the time required for a result.

*Method.*—Pure cultures of the same bacilli were grown in tubes of plain dextrose broth for 24 and 72 hours under identical conditions, and guinea-pigs were injected intraperitoneally and subcutaneously with doses proportioned to body-weight, in accordance with our general rule.

The results, shown in Tables 6 and 9, may be summarized as follows: Of 27 cultures tested for virulence by these methods, 74% yielded positive results in subcutaneous injection of 72-hour plain-dextrose-broth growths, as compared with 63% positive reactions in intraperitoneal injection of corresponding doses of 24-hour growths in the same medium. In the case of 7 cultures (Table 6, Nos. 4, 7, and 10; Table 9, Nos. 1, 4, 5, and 9) the positive results with both methods were observed at the same intervals after injection; for 5 cultures (Table 6, Nos. 1 and 9; Table 9, Nos. 3, 8, and 9) the subcutaneous method yielded an earlier result; for only 2 cultures (Table 6, Nos. 5 and 8) did the intraperitoneal method yield a quicker result. These results show definitely the superiority of the routine subcutaneous test with 72-hour cultures over the intraperitoneal injection of 24-hour cultures.

*The Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour, and Intraperitoneal Injection of 24-Hour, Serum-Dextrose-Broth Cultures.*—As the addition of serum to broth enhances the growth of diphtheria bacilli, we have tested the comparative delicacy of intraperitoneal injections with serum-broth cultures, the technic being the same as that described for the plain-broth cultures. The results are shown in Table 7.

In the case of 8 cultures tested, including granular, long solid, and short solid types of bacilli, the results were about equal with both methods in that both showed virulent bacilli for 50% of the cultures.

TABLE 7  
COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR,  
AND INTRAPERITONEAL INJECTION OF 24-HOUR, SERUM-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Throat.....	15	Granular (C).....
2	Throat.....	3	Long solid (C <sub>2</sub> ).....
3	Ear.....	18	Granular (C).....
4	Throat.....	16	Granular (C).....
5	Ear.....	28	Granular (C).....
6	Nose.....	21	Short solid (D <sub>2</sub> ).....
7	Throat.....	19	Granular (C).....
8	Throat.....	17	Granular (C).....

TABLE 8  
COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRAPERITONEAL  
INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Nose.....	8	Short solid (D <sub>2</sub> ).....
2	Ear.....	19	Short solid (D <sub>2</sub> ).....
3	Ear.....	32	Short solid (D <sub>2</sub> ).....
4	Nose.....	20	Granular (C).....
5	Throat.....	31	Granular (C).....
6	Nose.....	30	Granular (C).....
7	Ear.....	39	Granular (C).....
8	Ear.....	21	Granular (C).....
9	Throat.....	19	Granular (C).....
10	Throat.....	25	Granular (C).....
11	Throat.....	19	Granular (C).....
12	Throat.....	19	Granular (C).....
13	Nose.....	28	Short solid (D <sub>2</sub> ).....
14	Throat.....	18	Long solid (C <sub>2</sub> ).....
15	Throat.....	42	Short solid (D <sub>2</sub> ).....

TABLE 9  
COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRAPERITONEAL  
INJECTION OF 72-HOUR, AND INTRAPERITONEAL INJECTION OF 24-HOUR,  
PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus	72-Hour Plain-Dextrose-Broth Cultures (Subcutaneously)					
				Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
1	Ear	25	Granular (C)	260	1.3	+	0	0	0
2	Nose	125	Granular (C)	256	1.3	—	+	0	0
3	Nose	40	Granular (C)	243	1.2	—	+	0	0
4	Ear	27	Granular (C)	256	1.3	+	0	0	0
5	Ear	31	Granular (C)	261	1.3	+	0	0	0
6	Nose	30	Long solid (C <sub>2</sub> )	241	1.2	—	+	0	0
7	Throat	14	Long solid (C <sub>2</sub> )	300	1.5	—	+	0	0
8	Nose	17	Granular (C)	273	1.3	+	0	0	0
9	Throat	21	Granular (C)	248	1.3	—	+	0	0
10	Nose	18	Long solid (C <sub>2</sub> )	260	1.3	—	—	—	—
11	Throat	4	Long solid (C <sub>2</sub> )	263	1.3	—	—	—	—
12	Throat	23	Granular (C)	269	1.3	—	—	—	—

TABLE 7—Continued

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR,  
AND INTRAPERITONEAL INJECTION OF 24-HOUR, SERUM-DEXTROSE-BROTH CULTURES

72-Hour Serum-Dextrose-Broth Cultures (Subcutaneously)						24-Hour Serum-Dextrose-Broth Cultures (Intraperitoneally)					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
255	1.3	—	+	0	0	252	1.3	—	+	0	0
292	1.5	—	—	—	—	280	1.4	—	—	—	—
268	1.3	—	—	+	0	275	1.4	—	+	0	0
276	1.4	—	+	0	0	294	1.5	—	+	0	0
295	1.5	—	—	—	Toxic	249	1.3	—	—	—	+
300	1.5	—	—	—	—	270	1.4	—	—	—	—
280	1.4	—	—	+	0	285	1.4	—	+	0	0
276	1.4	—	—	—	+	300	1.5	+	—	—	—

TABLE 8—Continued

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRAPERITONEAL  
INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES

72-Hour Plain-Dextrose-Broth Cultures (Subcutaneously)						72-Hour Serum-Dextrose-Broth Cultures (Intraperitoneally)					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
280	1.4	—	—	—	—	274	1.4	—	—	—	—
260	1.3	—	—	—	—	263	1.3	—	—	—	—
305	1.5	—	—	—	—	295	1.5	—	—	—	—
240	1.2	—	+	0	0	250	1.25	—	+	0	0
257	1.3	—	+	0	0	256	1.3	—	—	+	0
315	1.6	—	—	+	0	264	1.3	—	—	+	0
284	1.4	—	—	—	+	262	1.3	—	—	—	—
268	1.3	—	+	0	0	276	1.4	—	—	—	Toxic
266	1.3	—	+	0	0	286	1.4	—	+	0	0
294	1.5	—	+	0	0	268	1.3	—	+	0	0
254	1.3	—	—	+	0	270	1.4	—	+	0	0
276	1.4	—	—	—	—	272	1.3	—	—	—	—
294	1.5	—	—	—	—	254	1.3	—	—	—	—
300	1.5	—	—	—	Toxic	318	1.6	—	—	—	—
284	1.4	—	—	—	—	290	1.5	—	—	—	—

TABLE 9—Continued

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRAPERITONEAL  
INJECTION OF 72-HOUR, AND INTRAPERITONEAL INJECTION OF 24-HOUR,  
PLAIN-DEXTROSE-BROTH CULTURES

72-Hour Plain-Dextrose-Broth Cultures (Intraperitoneally)						24-Hour Plain-Dextrose-Broth Cultures (Intraperitoneally)					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, s.s.	1 Da.	2 Da.	3 Da.	4 Da.
300	1.5	+	0	0	0	287	1.4	+	0	0	0
300	1.5	—	—	—	—	300	1.5	—	—	—	—
280	1.4	—	—	+	0	250	1.3	—	—	—	+
300	1.5	+	0	0	0	240	1.2	+	0	0	0
295	1.5	+	0	0	0	240	1.2	+	0	0	0
246	1.2	+	0	0	0	250	1.3	—	+	0	0
282	1.4	—	—	+	0	240	1.2	—	+	0	0
272	1.4	—	—	+	0	260	1.3	—	—	—	+
261	1.3	+	0	0	0	240	1.2	—	+	0	0
249	1.3	—	—	—	—	280	1.4	—	—	—	—
244	1.2	—	—	—	—	260	1.3	—	—	—	—
246	1.3	—	—	—	—	280	1.4	—	—	—	—

In the case of 4 cultures (Nos. 3, 5, 7, and 8) the intraperitoneal injection of 24-hour serum-broth cultures yielded quicker or more positive results than did the subcutaneous injection of 72-hour plain-broth cultures. As shown by these tests, therefore, the intraperitoneal injection of 24-hour serum-broth cultures as a method, is equal to the subcutaneous injection of 72-hour cultures and somewhat superior, in that less time is required for the conduct of the virulence test.

*Comparative Virulence of Diphtheria Bacilli in Subcutaneous and Intraperitoneal Injection of 72-Hour Plain-Dextrose-Broth Cultures.*—Further to study the possible superiority of the intraperitoneal over the subcutaneous route for conducting virulence tests, we tested a number of cultures by both methods, the technic being similar to that already described. The results are given in Tables 8 and 9.

TABLE 10

A SUMMARY OF RESULTS REGARDING THE VIRULENCE OF DIPHTHERIA BACILLI AS DETERMINED BY VARIOUS METHODS

Total Examined	Culture Medium	Hours of Incubation	Route of Injection	Percentage of Positive Results
37	Plain Dextrose Broth.....	72	Subcutaneous.....	86.5
22	Plain Dextrose Broth.....	72	Subcutaneous.....	63.6
8	Serum Dextrose Broth.....	72	Subcutaneous.....	62.5
27	Plain Dextrose Broth.....	72	Subcutaneous.....	74.0
8	Serum Dextrose Broth.....	72	Subcutaneous.....	75.0
27	Plain Dextrose Broth.....	72	Subcutaneous.....	63.0
9	Plain Dextrose Broth.....	72	Subcutaneous.....	45.5
8	Plain Dextrose Broth.....	72	Subcutaneous.....	50.0

In the case of 27 cultures tested, including granular, long solid and short solid types, the subcutaneous injection of 72-hour growths in plain dextrose broth yielded 63% positive results as compared with 55.5% positive results with the intraperitoneal injection of 72-hour plain-dextrose-broth cultures of the same bacilli cultivated under the same conditions. Usually the positive results were observed just as early with the subcutaneous, as with the intraperitoneal, injections. These results indicate that the intraperitoneal injection of plain-dextrose-broth cultures offers no advantages over the subcutaneous route.

#### SUMMARY AND CONCLUSIONS

A summary of these comparative virulence tests is shown in Table 10. The results of the study and the conclusions drawn may be stated as follows:

The intracutaneous injection of 0.1 c.c. of 72-hour plain-dextrose-broth cultures of diphtheria bacilli proved inferior to the subcutaneous injection of the same cultures into guinea-pigs, weighing from 250 to 300 gm., in dose corresponding to 0.5% of the body weight expressed in cubic centimeters. The former method yielded 64.9% positive results, as compared with 86.5% with the latter method.

It is more difficult to read and interpret the results with the intracutaneous method than with the subcutaneous method.

Of all the methods employed, that of subcutaneous injection of suspensions of 24-hour Loeffler cultures in normal salt solution yielded the best results. This method yielded 69.6% positive results as compared with 65.1% positive results, with the subcutaneous injection of 72-hour plain-dextrose-broth cultures.

TABLE 10—*Continued*

A SUMMARY OF RESULTS REGARDING THE VIRULENCE OF DIPHTHERIA BACILLI AS DETERMINED BY VARIOUS METHODS

Culture Medium	Hours of Incubation	Route of Injection	Percentage of Positive Results
Plain Dextrose Broth.....	72	Intracutaneous.....	64.9
Loeffler.....	24	Subcutaneous.....	68.1
Loeffler.....	24	Subcutaneous.....	62.5
Plain Dextrose Broth.....	24	Intraperitoneal.....	63.0
Serum Dextrose Broth.....	24	Intraperitoneal.....	75.0
Plain Dextrose Broth.....	72	Intraperitoneal.....	55.5
Plain Dextrose Broth.....	9 days	Subcutaneous.....	66.6
Serum Dextrose Broth.....	72	Subcutaneous.....	75.0

The subcutaneous injection of 72-hour serum-broth cultures yielded the same percentage (62.5) of positive reactions as the subcutaneous injection of 24-hour Loeffler cultures, but the latter is a superior method, as it consumes less time, while being just as delicate and positive in its results.

Twenty-four-hour Loeffler cultures in subcutaneous injection yielded the same results as 9-day plain-dextrose-broth cultures.

Serum-dextrose-broth cultures yielded a higher percentage of positive results (75%) than did plain-dextrose-broth cultures (50%) when both were cultivated for the same length of time and injected subcutaneously in equal dosage.

The subcutaneous injection of 72-hour plain-dextrose-broth cultures proved superior to the intraperitoneal injection of 24-hour and 72-hour plain-dextrose-broth cultures. With serum-dextrose-broth cultures, however, the intraperitoneal injection of 24-hour growths was

equal to, if not slightly superior to, the subcutaneous injection of 72-hour cultures.

From the standpoints of delicacy and of time required, the subcutaneous injection of 24-hour Loeffler cultures after the method described, yielded the best results.

The subcutaneous injection of 72-hour serum-dextrose-broth cultures yielded equally good results, but this method requires more time for the conduct of a virulence test; likewise, the intraperitoneal injection of 24-hour serum-dextrose-broth cultures yielded good results, but this method is less to be preferred than subcutaneous inoculation, because with the latter, local inflammatory changes are more easily detected.

# A STUDY OF ACID-PRODUCTION BY DIPHTHERIA BACILLI \*

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While investigators are in general accord regarding the power of virulent diphtheria bacilli to split dextrose with the formation of organic acids, there is considerable difference of opinion regarding the action of these bacilli on other carbohydrates. It is generally agreed that avirulent bacilli are less able than virulent bacilli to produce acids from carbohydrates or even lack this power entirely, and various observers have therefore advocated fermentation tests for the differentiation of various diphtheria-like bacilli from one another and from the true diphtheria bacillus.

## HISTORICAL REVIEW

The earliest work on this subject consisted in the titration of cultures for acidity, the cultures having been grown in broth of known titer for varying periods of time. In this manner Biggs, Park, and Beebe<sup>1</sup> early observed and drew attention to the varying powers of diphtheria bacilli in the production of acid in dextrose broth, and differentiated between true and pseudodiphtheria bacilli according to their virulence for the guinea-pig and their power of acid-production in this medium. This power of true diphtheria bacilli to produce acids from dextrose was likewise observed by Theobald Smith,<sup>2</sup> who drew attention to the absence of acid-production in broths containing the disaccharids lactose and saccharose. L. Martin<sup>3</sup> reported that true diphtheria bacilli were able to split dextrose, galactose, levulose, and to a lesser degree saccharose and glycerin; he observed negative results with lactose, maltose, raffinose, arabinose, dulcitol, and mannitol.

Shortly thereafter several investigators undertook to differentiate between virulent and nonvirulent diphtheria bacilli and the pseudobacillus of Hofmann-Wellenhof by means of tests for acidity in broth containing dextrose and other carbohydrates. While, as previously stated, practically all investigators have found that the true diphtheria bacillus produces relatively large amounts of acids from dextrose, there is not by any means a uniformity of opinion regarding the value of this test in differentiating among the various kinds of diphtheria bacilli. The investigations of Peters,<sup>4</sup> Kurth,<sup>5</sup> Schmitz,<sup>6</sup> and others

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<sup>1</sup> Centralbl. f. Bakteriöl., 1895, 17, p. 765.

<sup>2</sup> Tr. Assn. Am. Phys., 1896, 11, p. 37.

<sup>3</sup> Ann. de l'Inst. Pasteur, 1898, 12, p. 26.

<sup>4</sup> Deutsch. med. Wchnschr., 1897, 23, p. 133.

<sup>5</sup> Ztschr. f. Hyg. u. Infectiouskrankh., 1898, 28, p. 409.

<sup>6</sup> Ibid., 1913, 75, p. 513.

have led them to minimize the value of these acid-production tests in differentiating among the members of the group of diphtheria bacilli, while Bronstein and Grünblatt,<sup>7</sup> Riemsdijk,<sup>8</sup> and others regard acid-production from glucose an absolute criterion of differentiation among diphtheria and pseudodiphtheria bacilli.

With the advent of the serum-water medium of Hiss containing 1% of carbohydrate with litmus as an indicator, a means was afforded for applying these acid-production tests with a number of carbohydrates on a large scale and in a manner much simpler than that of the titration method. Using this medium, Knapp<sup>9</sup> studied 27 cultures of diphtheria-like bacilli and reported very favorably upon it as a means of differentiating *Bacillus hofmanni* and *Bacillus xerosis* from the true diphtheria bacillus. Similar results were reported by Zinsser.<sup>10</sup> Hamilton and Horton<sup>11</sup> were unable to confirm Knapp's results, but found that the true diphtheria bacillus always produced acid in dextrin broth.

Graham-Smith,<sup>12</sup> using the Hiss medium and a number of different carbohydrates, studied 39 races of diphtheria bacilli, and found marked acid-production with glucose, levulose, galactose, maltose, and dextrin; variable results with lactose and saccharose; and no acid at all with mannite. Differences were found between the series grown on plain broth plus the carbohydrate and that grown on the same carbohydrate in Hiss' medium, probably due to the fact that some of the bacilli grew poorly on the former but well on the latter.

Kolmer,<sup>13</sup> in a study of acid-production with 72 cultures that had been tested for virulence by guinea-pig inoculation, found that virulent bacilli produced acid most frequently with dextrose, and next most frequently with dextrin. With the remaining sugars the results varied. Cultures proving nonvirulent that produced acid with some of the sugars, especially dextrose and dextrin, were regarded as nonvirulent diphtheria bacilli. The sugar tests were found of most practical value in dealing with the solid types so frequently found in normal noses and throats.

More recently Hine<sup>14</sup> has advocated these acid-production tests as a means of differentiation, and on this basis he divides diphtheria bacilli into 3 groups. The 1st group, comprising the true Klebs-Loeffler bacilli, produces acid with dextrose, maltose, and dextrin, and generally with lactose, but not with saccharose; the 2nd group, comprising bacilli morphologically indistinguishable from the true bacillus and usually observed in cultures from the skin and adjacent mucous membranes, produces acid with glucose and saccharose, and frequently with maltose and lactose, but not with dextrin; the bacilli of the 3rd group produce acid with dextrose, saccharose, maltose, and dextrin, but not with lactose. He reports that he has never found any but true virulent diphtheria bacilli to produce acid with dextrose and dextrin but not with saccharose, and he advocates these tests as a practical means of differentiation superior to the guinea-pig-inoculation test. *Bacillus hofmanni* in all these investigations was found unable to produce acids with any of the sugars mentioned.

<sup>7</sup> Centralbl. f. Bakteriöl., I, O., 1902, 32, p. 425.

<sup>8</sup> Ibid., 1914, 75, p. 229.

<sup>9</sup> Jour. Med. Research, 1904, 7, p. 475.

<sup>10</sup> Ibid., 1907-1908, 17, p. 227.

<sup>11</sup> Jour. Infect. Dis., 1906, 3, p. 128.

<sup>12</sup> The Bacteriology of Diphtheria, 1908, p. 157.

<sup>13</sup> Jour. Infect. Dis., 1912, 11, p. 56. Arch. Pediat., 1912, 29, p. 94.

<sup>14</sup> Jour. Path. and Bacteriol., 1913-1914, 18, p. 75.

During the past 15 months, in connection with the routine animal-inoculation tests for virulence, we have studied a number of cultures from various sources, the object being (1) to note the acid-production with various carbohydrates by cultures of diphtheria bacilli from various sources, of varying morphology, and of known virulence as determined by animal-inoculation tests; and (2) to ascertain the practical value of these tests in aiding differentiation among the members of the diphtheria group.

#### MATERIALS AND METHODS OF STUDY

*Cultures.*—In all, 392 cultures of diphtheria bacilli have been studied. Of these, 233 were isolated from cultures of the throat; 83 from the nose; 58 from the ear in cases of suppurative otitis media; 12 from the skin; and 6 from the eye. All the cultures were from patients in the wards of the Philadelphia hospital for contagious diseases, most of them from persons suffering with diphtheria. All cultures had been tested for virulence by the guinea-pig-inoculation test. The technic employed and the results observed are given in separate papers dealing with that subject.<sup>15</sup> General toxemia and local edema at the site of inoculation were accepted as evidences of virulence; absence of toxemia and local reaction over a period of 4 days were regarded as indicating absence of virulence. However, if the guinea-pig showed definite edema at the site of inoculation, even tho without the well-marked general toxemia and without succumbing by the 4th day, the result was interpreted as positive, that is, as indicating virulence of the bacilli.

The morphology of the bacilli was recorded according to Wesbrook's classification. While cultures of granular or solid types of the bacilli tend to preserve their group characteristics, we have found so many variables in a culture of a granular type of bacillus, that no attempt has been made to classify the sugar reactions, according to the subvarieties, as bacilli of the A, B, C, D, etc., types. Instead, all granular types are listed under the one heading "granular," as we have included only the common varieties. The solid varieties were divided into 2 main types; namely, the long solid bacilli corresponding to the C<sub>2</sub> type of Wesbrook; and the short solid bacilli corresponding to the D<sub>2</sub> types.

*Technic.*—Hiss's serum-water medium, containing 1% of sugar and colored with litmus or azolitmin, was employed in most of this work.

Cultures of bacilli were grown in plain or glucose broth for 3 days before transplantation to the Hiss medium. In this manner all cultures were trained to grow in a fluid medium before subculturing—a matter of importance especially in relation to the rapidity, and to some extent the degree, of acid-production and coagulation in the Hiss medium.

A number of sugars were used with each culture. All cultures were grown for 4 days at 37 C. and the results recorded according to acid-production and coagulation. With many of the cultures the results were recorded each day for 4 days. When changes occurred in the medium, the culture was examined for purity; this was found to be absolutely necessary in guarding against occasional contamination.

<sup>15</sup> Kolmer and Moshage: Jour. Infect. Dis., 1916, 19, p. 1. Kolmer, Woody, and Moshage: Am. Jour. Dis. Child., 1916, 11, p. 257.

The following carbohydrates were used:

Monosaccharids	{ Dextrose Levulose Galactose	Disaccharids	{ Maltose Lactose Saccharose
Trisaccharids	{ Raffinose Arabinose	Polysaccharids	{ Dextrin Alcohol mannite

RESULTS

*Acid-Production in Hiss Serum-Water Media.*—The results obtained are summarized in Tables 1 and 2 according to the type of bacillus in the culture and the results of guinea-pig-inoculation tests for virulence.

TABLE 1  
ACID-PRODUCTION IN HISS SERUM-WATER MEDIA

Sugars	Virulent Cultures				Nonvirulent Cultures				
	Total	Num- ber Pro- ducing Acid	No Change	Per- centage Produc- ing Acid	Total	Num- ber Pro- ducing Acid	No Change	Per- centage Produc- ing Acid	
ACID-PRODUCTION BY GRANULAR TYPES OF DIPHTHERIA BACILLI (MOSTLY TYPE C)									
Dextrose.....	70	68	2	97	31	29	2	94	
Levulose.....	43	38	5	90	7	6	1	86	
Galactose.....	64	58	6	91	20	18	2	85	
Maltose.....	55	28	27	51	18	8	10	42	
Lactose.....	64	20	44	31	14	4	10	29	
Saccharose.....	73	28	45	40	43	14	29	32	
Raffinose.....	40	8	32	20	19	2	17	10	
Arabinose.....	24	2	22	4	18	1	17	5	
Dextrin.....	81	73	8	90	22	19	3	86	
Mannite.....	77	0	77	0	47	0	47	0	
ACID-PRODUCTION BY LONG SOLID TYPES OF DIPHTHERIA BACILLI									
Dextrose.....	48	47	1	98	87	83	4	95	
Levulose.....	11	10	1	90	29	26	3	90	
Galactose.....	60	49	11	82	115	93	22	81	
Maltose.....	34	14	20	41	29	9	20	31	
Lactose.....	62	30	32	48	118	42	76	35	
Saccharose.....	54	4	50	7	112	7	105	6	
Raffinose.....	18	2	16	11	78	6	72	7	
Arabinose.....	18	1	17	5	15	0	15	0	
Dextrin.....	75	37	38	49	138	54	86	40	
Mannite.....	74	0	74	0	140	0	140	0	
ACID-PRODUCTION BY SHORT SOLID TYPES OF DIPHTHERIA BACILLI (MOSTLY D <sub>2</sub> )									
Dextrose.....				30		2		28	6
Levulose.....				30		0		30	0
Galactose.....				31		1		30	3
Maltose.....				30		1		29	3
Lactose.....				30		0		30	0
Saccharose.....				27		0		27	0
Raffinose.....				30		0		30	0
Arabinose.....				12		0		12	0
Dextrin.....				27		0		27	0
Mannite.....				30		0		30	0

TABLE 2

SUMMARY OF THE PERCENTAGES OF CULTURES OF THE VARIOUS TYPES OF DIPHTHERIA BACILLI WHICH PRODUCED ACID

Sugars	Granular Types		Long Solid Types		Short Solid Types Nonvirulent
	Virulent	Nonvirulent	Virulent	Nonvirulent	
Dextrose.....	97	94	98	95	6
Levulose.....	90	86	90	90	0
Galactose.....	91	85	82	81	3
Maltose.....	51	42	41	31	3
Lactose.....	31	29	48	35	0
Saccharose.....	40	32	7	6	0
Raffinose.....	20	10	11	7	0
Arabinose.....	4	5	5	0	0
Dextrin.....	90	86	49	40	0
Mannite.....	0	0	0	0	0

Virulent diphtheria bacilli are more prone to ferment carbohydrates than are nonvirulent bacilli; this is generally true of all types independently of source and morphologic character. Acid-production by diphtheria bacilli therefore runs somewhat parallel with toxin-production; as shown in the tables, however, it is commonly observed that a culture nonvirulent for guinea-pigs may be capable of splitting carbohydrates. Further study on the relationship of the carbohydrate-splitting ferments to the soluble toxins of diphtheria bacilli is given in another communication.<sup>17</sup>

Acid-production generally was found most frequently with dextrose, levulose, galactose, and dextrin.

The granular or beaded types of bacilli generally yielded slightly higher percentages of acid-production than did the long solid types.

The majority of the cultures of the short solid thick D<sub>2</sub> types of bacilli failed to produce acids with any of the carbohydrates used in this study. Bacilli of this type resemble the Hoffmann-Wellenhof bacillus rather closely in morphologic and biologic characters. The typical pseudodiphtheria, or Hofmann, bacillus does not ferment any sugar, and for this reason we have found acid-production tests of most value in identifying this bacillus. A bacillus of this morphology, fermenting dextrose and proving nonvirulent for the guinea-pig, we regard as a true nonvirulent diphtheria bacillus.

Cultures of virulent bacilli of the granular or beaded types show most constant and vigorous acid-production; the short solid nonvirulent races show little or no tendency toward acid-production. Between these extremes is a group of bacilli with valuable fermentative powers.

<sup>17</sup> Kolmer and Moshage: Jour. Infect. Dis., 1916, 19, p. 28.

Considerable interest is attached to the fermentation of saccharose; in our experience about 36% of cultures of granular bacilli and 6% of the long solid types of bacilli produced acids with this sugar. This property is by no means confined to cultures of diphtheria-like bacilli isolated from the conjunctiva (*B. xerosis*), so that, contrary to certain statements, the use of this sugar proved to be of little value in differentiating between true diphtheria bacilli and *B. xerosis* on the basis that the former is unable to cleave saccharose while the latter possesses this property.

In common with other observers we have not encountered cultures of diphtheria bacilli capable of definitely cleaving mannite. Occasionally acid has been produced, but further study has invariably shown the culture to be contaminated. With prolonged incubation a small percentage of cultures turn the culture medium a reddish tint, but evidences of definite acid-production and coagulation in the Hiss medium have not been observed.

TABLE 3  
RAPIDITY OF ACID-PRODUCTION BY DIPHTHERIA BACILLI WITH VARIOUS CARBOHYDRATES

Sugars	Total	Percentage of Acid Produced During a Period of 4 Days			
		1st Day	2d Day	3d Day	4th Day
Dextrose.....	131	73	27	..	..
Levulose.....	78	63	29	8	..
Galactose.....	150	62	26	10	2
Maltose.....	92	46	40	14	..
Lactose.....	79	35	60	3	2
Saccharose.....	85	62	25	10	3
Raffinose.....	48	30	20	..	..
Arabinose.....	10	35	60	5	..
Dextrin.....	131	65	30	5	..
Mannite.....	82	0	0	0	0

*Rapidity of Acid-Production.*—As shown in Table 4, the majority of cultures produced sufficient acid definitely to affect litmus in the first 24 hours of incubation. Rapidity of acid-production is probably parallel to some extent with rapidity of growth and multiplication of the bacilli; the growth of our cultures in a fluid medium before transference to the Hiss medium influenced the rate of acid-production during the first day or two. While cultivation of the bacilli for periods of time exceeding 4 days may yield higher percentages of positive results, in our experience cultures capable of splitting a carbohydrate in the Hiss medium show this property within 4 days in the majority of instances.

Rapidity of acid-production, as shown in Table 3, was most apparent in media containing dextrose, levulose, galactose, and dextrin, these 4 carbohydrates being those which diphtheria bacilli are most able to cleave. Cultures failing to produce appreciable amounts of acid with these sugars in 24 hours usually do so within 48 hours and more certainly within 4 days.

These facts were further shown in a series of titrations for total acidity of cultures in neutral broths each containing 1% of one of the various carbohydrates. The results of titrations with two cultures are shown in Table 4.

TABLE 4  
RAPIDITY OF ACID-PRODUCTION BY DIPHTHERIA BACILLUS IN CARBOHYDRATE BROTHS

	Sugars	Results of Titrations for Acidity During a Period of 14 Days after Inoculation													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
A culture of a granular type isolated from the throat of a convalescent from diphtheria; virulent for guinea-pigs	Dextrose...	8	12	14	16	16	17	16	16	14	16	18	18	20	21
	Levulose...	8	14	20	23	24	25	24	24	26	26	26	26	25	24
	Galactose..	6	15	16	19	20	22	20	20	19	20	20	20	20	20
	Lactose....	4	7	12	13	14	17	14	14	12	12	11	12	10	8
	Saccharose	0.5	2	2	3	4	7	8	9	11	13	15	17	17	17
	Raffinose...	0.5	3	5	6	6	6	0	0	0	0	0	0	0	0
	Dextrin....	4	10	12	14	15	16	16	16	15	16	16	18	18	18
A culture of a long solid (C <sub>2</sub> ) type isolated from the throat of a convalescent; virulent for guinea-pigs	Mannite....	0	0	0	Tr.	Tr.	Tr.	0	0	0	0	0	0	0	0
	Dextrose...	7	9	12	10	13	12	12	14	14	16	15	15	15	16
	Levulose...	7	8	10	11	12	12	12	13	14	16	15	15	15	14
	Galactose..	2	4	3	4	4	4	4	6	9	11	11	11	11	14
	Lactose....	2	3	6	7	6	7	8	8	8	10	10	11	12	12
	Saccharose	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Raffinose...	0	Tr.	Tr.	0	0	0	0	0	0	0	0	0	0	0
	Dextrin....	2	6	10	12	11	11	13	14	14	16	15	15	15	14
	Mannite....	0	0	0	0	0	0	0	0	0	0	0	0	0	0

These titrations were made daily over a period of 2 weeks. All titrations were made with  $n/20$  sodium hydroxid, with phenolphthalein as the indicator. Duplicate titrations were conducted at incubator temperature with unfiltered cultures without boiling, and again after boiling for 2 minutes. The results shown in the tables were those observed with the latter method, the end reaction being taken as the first definite pink which held after the second boiling for a minute or two. The titrations of unheated cultures (not boiled) showed uniformly a slightly higher acidity, but otherwise the results were closely parallel with the results shown in the table.

The highest degrees of acidity occurred with dextrose, levulose, galactose, and dextrin; acid-production also occurred in the lactose broths but more slowly; a few cultures produced acid with saccharose and raffinose while others did not (as shown in Table 4). While

traces of acid were at times discovered in the mannite broths, the majority of the cultures failed entirely to cleave this carbohydrate.

*Acid-Production By Diphtheria Bacilli From Various Sources.*—Diphtheria bacilli of the same morphology isolated from different parts of the body vary in their power to produce acids in carbohydrate media. These differences, however, are apparently largely dependent on the question of virulence, as already pointed out. Bacilli from the throat and nose are usually more virulent than those from the ear; those derived from the skin and adjacent mucous membranes, such as the conjunctiva and genitalia, are usually nonvirulent and poor acid-producers. The general rule, however, that granular types of bacilli are likely to be more virulent than the short varieties is likewise observed in acid-production tests, in which they are more likely to produce acid.

Cultures of diphtheria bacilli from the throat generally possess most marked fermentative powers; those from the nose and ear are less active and those from the eye and skin (including the genitalia), least active.

*Coagulation of Hiss Serum Water.*—Coagulation of the serum in Hiss's medium is frequently observed, the time of its occurrence and the degree of coagulation being dependent on the time of production and the amount of organic acids formed. For example, with the monosaccharids coagulation is frequently observed on the 2nd day and sometimes on the 1st day of incubation; with other less readily fermented sugars coagulation is delayed and may be absent on the 4th day, even tho some acid has been produced.

#### PRACTICAL VALUE OF ACID-PRODUCTION TESTS

In our experience acid-production tests are of limited value in the classification of diphtheria bacilli. The difficulty of securing pure carbohydrates for these tests constitutes a disturbing factor which may account in part for the irregularities and inconstant results. From the practical standpoint, these tests have generally proved successful in the identification and classification of the Hofmann bacillus on the basis that it does not produce appreciable quantities of acids with carbohydrates. For the purpose of differentiating between virulent and non-virulent bacilli, these tests have failed to prove of any value; the typical virulent diphtheria bacillus produces acids most characteristically with dextrose, dextrin, levulose, and galactose, and the false types, or Hof-

mann's bacilli, produce no acids at all; but between these extremes is a large group of bacilli of varying morphology and from different sources which present varying and inconstant degrees of virulence and fermentative power.

Considerable interest is attached to cultures of diphtheria-like bacilli from the conjunctivae. Such bacilli from the normal eye, which are practically always nonvirulent, are well known under the name of *B. xerosis*. These micro-organisms are capable of fermenting saccharose, and we have found them capable of attacking other sugars as well, including dextrin and dextrose; as previously stated, we have not found that fermentation of saccharose and absence of fermentation of dextrin by these bacilli suffice to differentiate *B. xerosis* from the true diphtheria.

#### CONCLUSIONS

Acid-production by diphtheria bacilli in Hiss's serum-water-carbohydrate media is most marked with dextrose, dextrin, levulose, and galactose.

A small percentage of cultures from the throat and nose also produce acid with saccharose.

Nonvirulent diphtheria-like bacilli from the conjunctiva are capable of fermenting not only saccharose but also other sugars as well, including dextrin and dextrose.

Granular types of bacilli generally produce acids more frequently than do the solid types, and long solid types more frequently than the short varieties.

In general, the time of appearance and degree of acid-production from carbohydrates are somewhat parallel with the degree of virulence of the diphtheria bacilli.

Acid-production tests on different carbohydrates are not sufficiently regular to be depended on in determining the harmfulness of a given culture.

Acid-production tests are of most value in the study and classification of the solid varieties of the diphtheria-like bacilli; cultures proving nonvirulent for guinea-pigs and producing no acid with sugars are classified as the pseudo, or Hofmann's types; nonvirulent cultures producing acid with one or more sugars are regarded as non-virulent diphtheria bacilli.

# THE RELATION OF THE CARBOHYDRATE-SPLITTING FERMENTS TO THE SOLUBLE TOXINS OF DIPHTHERIA BACILLI \*

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Of considerable interest and importance is the possible relationship of the soluble toxins of which diphtheria toxin is a type, to the true enzymes, or unorganized-soluble ferments, elaborated by the vegetable cell, the activity of which is entirely independent of any of the life processes of the cell. There are several points of resemblance between the soluble toxins on one hand and the ferments, or enzymes, on the other. Both are products of the metabolism of living cells; both exhibit a latent period before manifesting their individual activities; both substances represent a method or means by which the organism attempts to modify its environment so as to render the surroundings suitable for its nutrition and growth; both show a strong affinity for their substrata; and first manifest their activity by combining with them; the activities of both seem to depend largely on the temperature to which they are exposed and both are usually affected by temperatures above 70 C.

The two great differences, however, between toxins and enzymes are the greater activity of the latter—even minute amounts of an enzyme having the power to split up or decompose large quantities of complex organic compounds, whereas when a toxin unites with a substance it loses its identity, and in its activity follows the law of multiple proportions—and the fact that antitoxins may be produced in large amounts whereas the production of antibodies to the enzymes, the true antiferments, is sharply limited.

It is well established that virulent diphtheria bacilli are invariably capable of splitting some carbohydrate, notably dextrose and dextrin, and it is also well proved that certain types of nonvirulent bacilli, notably *Bacillus hofmanni*, are unable to ferment any of the common carbohydrates, but between these opposite types may be found a large number of diphtheria bacilli apparently nonvirulent yet capable of fermenting certain carbohydrates in a rapid and characteristic manner.

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In other words, while we have never encountered a culture of diphtheria bacilli virulent for guinea-pigs that was totally without power of splitting some carbohydrate, we frequently have come upon cultures capable of exerting this ferment activity that were nonvirulent for guinea-pigs.

It would appear, therefore, that generally those bacilli capable of producing demonstrable amounts of soluble toxin, also produce carbohydrate-splitting ferments. Is there a possible relationship between the toxin and the carbohydrate-splitting process? Does the toxin itself behave as a ferment capable of acting on carbohydrates or is it entirely separate from and independent of these ferments?

We have studied these questions experimentally (1) by testing the carbohydrate-splitting powers of several potent bacteria-free diphtheria toxins; and (2) by cultivating virulent carbohydrate-splitting diphtheria bacilli in the presence of an excess of sterile preservative-free antitoxin in order to neutralize, as it were, the nascent toxin as quickly as produced. The results of our experiments support the view that diphtheria toxin itself apparently takes no part in the fermentation of carbohydrates, that soluble toxin and the carbohydrate-splitting ferments are separate secretory products of certain types of diphtheria bacilli.

#### EXPERIMENTS WITH BACTERIA-FREE TOXINS

The ferment activity of three different lots of filtered sterile diphtheria toxin was tested separately with dextrose, dextrin, and, in the case of Toxin 1, lactose—1% of each—in Hiss serum-water media.

Toxin 1 (obtained from Dr. A. P. Hitchens and Dr. C. P. Brown, Glenolden, Pa.) had been prepared about 2 months previously and preserved in the refrigerator with 0.5% tricresol. The L + dose was 0.82 c.c.

Toxin 2 (obtained from Dr. C. Y. White) had been prepared from the Park-Williams bacillus No. 8 and contained 0.4% tricresol. The M. L. D. was 0.01 c.c. In order to exclude any inhibitory influence of the preservative upon the possible carbohydrate-splitting properties of a soluble toxin, a freshly prepared product free of germicide or preservative was tested, but with negative results. The culture itself, however, fermented several carbohydrates, such as dextrin, galactose, and levulose, vigorously.

Toxin 3 was prepared by growing the Park-Williams bacillus No. 8 in broth for 5 days and filtering the living culture through a sterile

Berkefeld filter. The toxin was used at once. The M. L. D. was 0.03 c.c.

The technic of these experiments consisted in placing these sterile toxins, in amounts ranging from 0.1 c.c. to 2 c.c., into a series of sterile test tubes, adding sufficient of the Hiss medium to make the total volume in each tube about 5 c.c., and incubating the tubes for at least 6 days at 37 C. In no instance after 1, 2, 3, 4, 5, and 6 days was acid-production found to have occurred. (One toxin contaminated with a staphylococcus, which produced changes in the various media, is excluded in this report.)

#### EXPERIMENTS WITH NEUTRALIZED CULTURES

A number of cultures of virulent diphtheria bacilli were cultivated in Hiss media in the presence of increasing amounts of diphtheria anti-toxin designed to neutralize the toxin as quickly as produced by the bacilli, and acid-production studied with several carbohydrates. In this manner a further study was made of the possible relation of the soluble toxins to the processes of carbohydrate-fermentation.

The bacilli, which had been isolated from diphtheria patients, were of the granular varieties. Each was virulent for 300-gram guinea-pigs within a period of 3 days following subcutaneous injection of 1.5 c.c. of 3-day broth cultures.

A fresh antitoxin serum, free of preservative, was used. Subsequent titration of this serum showed that it contained from 1,000 to 1,100 units of antitoxin per cubic centimeter. This serum was diluted with sterile plain neutral broth 1:100, 1:10, and 1:5, and 2 c.c. of each dilution were placed in test tubes with 2 c.c. of Hiss serum-water medium containing 1% of a carbohydrate and colored with litmus. After the addition of these dilutions, the test tubes finally contained about 20, 200, and 400 units of antitoxin, respectively, and after adding 2 c.c. of undiluted antitoxin to 2 c.c. of the Hiss medium the 4th test tube contained 2,000 units, all in a total volume of 4 c.c.

All tubes were now inoculated with 0.1 c.c. of a 3-day broth culture of the diphtheria bacillus under study, and incubated at 37 C. for 4 or 5 days. Daily inspections were made and changes in the medium recorded.

After 5 days each tube of the series was examined for the presence of diphtheria bacilli and for purity of growth. (In every experiment the antitoxin dilutions were tested for sterility by culturing 2 c.c. of each in broth, and in all instances were found sterile.) After 4 or 5 days' incubation several cultures of each series were tested for free toxin by subcutaneously injecting 300-gram guinea-pigs with 1.5 c.c. of the growth from the antitoxin Hiss serum-water medium.

Table 1 shows this method of procedure in one experiment, with the results, which were fairly typical of all experiments.

TABLE 1  
CULTURE 7 \*

Tube	Hiss Serum Water Medium 2 c.c.	Units of Anti-toxin Added	Results					Examination of Culture After 5 Da.	Guinea-pig Inoculation Test
			1 Da.	2 Da.	3 Da.	4 Da.	5 Da.		
1	Dextrose	20	Acid	Acid	Acid Coagulation	Acid Coagulation	Acid Coagulation	Pure, good	Negative
2	Dextrose	200						Pure, good	Negative
3	Dextrose	400						Pure, good	Negative
4	Dextrose	2000						Few bacilli	Negative
5	Dextrin	20						Pure, good	Negative
6	Dextrin	200	.....	.....	.....	.....	.....	Pure, good	0
7	Dextrin	400						Pure, good	Negative
8	Dextrin	2000						Few bacilli	0
9	Broth	20	0	0	0	0	0	Sterile	0
10	Broth	400	0	0	0	0	0	Sterile	0

\* Isolated from the throat of a diphtheria convalescent. Granular, Type C. One and five-tenths cubic centimeters of a 3-day broth culture were fatal for a 300-gram guinea-pig in 3 days. Tubes of culture medium and antitoxin were inoculated with 0.1 c.c. of a 3-day broth culture.

Similar experiments were conducted with 6 other cultures, with almost identical results. Three cultures produced small amounts of acid in the presence of 2000 units of antitoxin after 3, 5, and 8 days respectively.

Our results show that the fermentation of these carbohydrates was in no way inhibited by the fact that the soluble toxins were neutralized as quickly as produced. In all instances the quantity of antitoxin added was sufficient to effect complete neutralization of the toxin, as determined by guinea-pig inoculation. When 2000 units of antitoxin were used, the growths of bacilli were relatively scant and we attribute the absence of fermentation within the 5-day limit in these tubes to the inhibitory influence of the serum on the growth of the bacilli.

That sufficient antitoxin to neutralize all toxin produced persisted in the Hiss serum-water-sugar cultures after an exposure to 37 C., was shown not only by the negative results of inoculation tests after 5 days, but also in another series of experiments, in which on separate occasions 3 virulent cultures of diphtheria bacilli were cultivated in Hiss serum-water-glucose medium in the presence of 100 units of sterile antitoxin, filtered through porcelain as soon as well-pronounced acid-production had occurred—usually about the second day—and 2 c.c. and 4 c.c. of the filtrate injected subcutaneously into guinea-pigs weighing 250 to 300 gm. The results of these animal tests were completely negative, the animals showing no edema or toxemia. Further than this the filtrate contained sufficient antitoxin to protect guinea-pigs against fatal doses of diphtheria toxin.

It would appear, therefore, that soluble diphtheria toxin takes no part in the fermentation of carbohydrates, this action being due to separate secretory products, or enzymes, of diphtheria bacilli.

Carbohydrate-fermentation in vitro by diphtheria bacilli does not run parallel with, or depend on, the vegetative vitality of the bacillus, that is, on its ability to survive and grow on artificial culture media, for pseudodiphtheria bacilli, as *B. hofmanni*, grow luxuriantly and yet are characterized by their lack of fermentative power for carbohydrates.

Acid-production by diphtheria bacilli, however, does run somewhat parallel to toxin-production and the degree of virulence. We have not so far found a culture both virulent for guinea-pigs and totally lacking in the power of fermenting some carbohydrate, whereas cultures totally unable to bring about fermentation have been found rather generally nonvirulent for animals. On the other hand, cultures of diphtheria bacilli nonvirulent for animals may be capable of splitting carbohydrates.

#### CONCLUSION

Carbohydrate-fermentation by diphtheria bacilli is dependent on secretory enzymic products of the diphtheria bacillus, but is independent of the soluble poison or toxin; these ferment-like carbohydrate-splitting products are most likely to be produced by toxin-producing bacilli.

# THE PROTEIN-SPARING ACTION OF UTILIZABLE CARBOHYDRATES IN CULTURES OF CERTAIN SUGAR-FERMENTING ORGANISMS \*

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Attention often has been called to the effect of the presence of various sugars on the protein metabolism of bacteria. It is a well-known fact that in cultures of certain organisms in the ordinary sugar-protein media, indol is not formed, gelatin is not liquefied, casein is not digested, and ammonia-production is greatly lessened.

Inasmuch as the decomposition of a carbohydrate is accompanied by the formation of more or less acid, and because the protein changes mentioned are commonly regarded as due to the action of one or more trypsin-like enzymes—that is, enzymes which do not act in acid media—some investigators have inclined to the belief that it is the acid resulting from the decomposition of the sugar, not the mere presence of the sugar itself, which alters the normal progress of the protein changes.

## HISTORICAL REVIEW

Wherry<sup>1</sup> has stated that the proteolytic ferments of bacteria are active only in a medium alkaline to litmus, that it takes but a small amount of acid to hinder their action, which is in accord with the behavior of trypsin, and that when carbohydrates which can be so fermented as to form acids, are present in gelatin, the liquefaction of the latter is inhibited. Glenn,<sup>2</sup> working with different strains of *B. proteus* and of *B. cloacae*, came to the same conclusion. Kuhn<sup>3</sup> concluded from his investigations that acids inhibit the liquefaction of gelatin, and Lehmann,<sup>4</sup> in whose laboratory Kuhn's work was done, stated that the inhibition of the liquefaction of gelatin is due to the trypsin-like nature—sensitiveness to acid—of this enzyme. Auerbach,<sup>5</sup> however, prevented the accumulation of the hydrogen ion by the use of magnesium oxid, and thus showed that Lehmann's explanation was no longer adequate. Jordan<sup>6</sup> noted that "the production of a gelatin-liquefying enzyme is not very dependent upon the reaction of the culture medium. It is probable that the reaction is of importance only in so far as it influences the general conditions of growth of the micro-organism."

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<sup>1</sup> Jour. Infect. Dis., 1905, 2, p. 317.

<sup>2</sup> Centralbl. f. Bacteriol., I, O., 1913, 58, p. 481.

<sup>3</sup> Arch. f. Hyg., 1891, 13, p. 70.

<sup>4</sup> Grundriss und Atlas der Bacteriol., 1890, p. 57.

<sup>5</sup> Arch. f. Hyg., 1897, 31, p. 311.

<sup>6</sup> Biological Studies by the Pupils of William Thompson Sedgwick, 1906.

Hirschler,<sup>†</sup> before Auerbach, had shown that phenol, skatol, cresol, indol, and the oxy-acids are not formed in mixed cultures when starch, cane sugar, dextrin, glycerin, or calcium lactate are present, even tho the acid reaction is kept down, as it was in his experiments, by calcium carbonate. He expressed himself on this point as follows: "It is quite probable that the acid resulting from the decomposition of the carbohydrates is the cause of the absence of protein decomposition, but up to the present, we lack proof that this is alone responsible for the hindering effect of the carbohydrates. It is quite probable that the presence of substances which are more easily decomposed than are proteins, is the cause of the absence of protein decomposition. Moreover, it is possible that these easily decomposed substances, as sugar, glycerin, etc., call forth a vigorous development of those varieties (of mixed culture) which decompose these substances, and this in turn influences unfavorably the other varieties which can decompose only the proteins."

Kendall, Day, and Walker,<sup>‡</sup> in observations extending over a number of years, have demonstrated the protein-sparing action of utilizable carbohydrates in bacterial metabolism. They regard this protein-sparing action as due, not to the inhibiting action of the acid resulting from the decomposition of the carbohydrate, but to the fact that utilizable sugars serve the fuel requirements of bacteria better than do proteins. Since the fuel requirements of bacteria are so much in excess of their structural requirements, it is evident that a utilizable sugar, by lending itself more readily to oxidation and energy-giving reactions, simply diverts the attack of the bacteria from the protein constituents of the medium, so that the products of decomposition on the protein side of their diet are almost entirely wanting, a slight ammonia-production being the only one in evidence and that merely representing changes in whatever part of the protein is necessary for the structural purposes of the bacteria.

In consideration of this lack of agreement in opinions as to how the presence of the utilizable sugar affects the protein metabolism of the bacteria, the following series of experiments was undertaken to determine (1) in what respects the proteolytic enzymes of bacteria are tryptic in nature, (2) the nature of the conditions which prevent the formation of the enzymes in sugar-protein media, and (3) the nature of the conditions which prevent the action of the enzyme after it has appeared in the cultures.

The conclusions presented are based on observations made on media containing, unless otherwise stated, 1% peptone and 0.5%  $\text{NH}_4\text{Cl}$ . *Bacillus proteus*, as a type of the proteolytic sugar-fermenting group of organisms, was used for inoculations. A sufficient number of other organisms of this type—*B. mesentericus*, *B. prodigiosus*, the spirillum of Asiatic cholera—and other combinations of nutrient media were also used so that the conclusions reached might not be open to objection as applying only to *B. proteus* in media of relatively simple composition.

<sup>†</sup> Ztschr. f. physiol. Chem., 1886, 10, p. 306.

<sup>‡</sup> Jour. Am. Chem. Soc., 1914, 36, p. 1966.

The various reactions of the media used in this work are expressed in terms of the normality system. In view of the fact that the percentage of dissociation of a given normality of a given acid in a medium of given composition is so widely different from that of the same normality of the same acid in water solution, or in a medium of different composition, the distinction between titratable acid and "hydrogen-ion concentration" must not be overlooked, for under ordinary cultural conditions it is the dissociated hydrogen, not the titratable hydrogen, which modifies the progress of changes produced in the media by bacterial enzymes. For example, the maximal amount of titratable acid produced by *B. bulgaricus* in rennet whey is N/50. The maximal amount produced in skimmed milk is N/6—over 8 times as much as in rennet whey. Yet a measurement of the hydrogen-ion concentration of these two cultures by the electrometric method shows identical degrees of acidity (if active, or dissociated, hydrogen is meant)—a concentration representing a definite and fixed limit for each strain of the organism, and, provided normal growth is obtained, affected neither by the protein content nor by the initial reaction of the medium. The great difference in the amounts of titratable acid in the two media in this case is due to a difference between the so-called buffer content of the whey and that of the skimmed milk. The excess of protein in the skimmed milk simply represses the dissociation of the hydrogen ion of the acids of the carbohydrate-fermentation, thereby protecting the bacteria over a longer period of their vegetative activity, and permitting a greater accumulation of these acids in the molecular, or titratable, form.

A measurement of the titratable acid is, however, an indirect measurement of the dissociated hydrogen, provided the measurement is made each time for a given acid in a medium of approximately constant buffer content. No attempt has been made, therefore, to draw conclusions regarding the concentration of hydrogen ion in one medium, by measuring a like amount of titratable acid in another medium, except in one especially noted case, in which the buffer values of the two media were sufficiently close to warrant the conclusions there reached.

## I

### THE METHOD OF PREPARATION OF THE PROTEOLYTIC ENZYME OF *B. PROTEUS*

One liter of a 7-day-old  $\text{NH}_4\text{Cl}$ -peptone culture of *B. proteus* was passed through a Berkefeld filter to remove the bacterial bodies. The water of the filtrate was then evaporated off at room temperature over sulfuric acid in a

sterile desiccator. The dry residue was rubbed up with 98% alcohol. A large part of the peptone collected as a gummy mass, which was then removed with the stirring rod. Small flocculent particles floating in the alcohol were separated from the  $\text{NH}_4\text{Cl}$  (which is soluble in 98% alcohol) by filtering through filter paper. The alcoholic filtrate when evaporated left a residue (mainly consisting of  $\text{NH}_4\text{Cl}$ ) which was almost entirely without effect on gelatin. The result showed also that the enzyme is practically insoluble in alcohol. Thus, one after another, the bacteria, the water, a large part of the peptone, and the  $\text{NH}_4\text{Cl}$  were removed from the flocculent material on the filter paper. This substance when removed and dried gave a fine white powder, which liquefied carbogelatin in concentrations of 1:10,000. Its activity remained apparently undiminished after months of exposure to the action of light and air. In this respect it differs from trypsin.

#### THE DIGESTION OF OTHER PROTEINS WITH THE PROTEOLYTIC ENZYME OF *B. PROTEUS*

*Casein*.—Five milligrams of this powdered enzyme were added to 5 c.c. of a solution containing 0.5% phenol, 0.1% powdered casein, and 0.1%  $\text{Na}_2\text{CO}_3$ . A 5-c.c. tube of casein solution without enzyme was used as a control. After 6 hours at 37 C. the contents of the two tubes were mixed with a few drops of acetic acid (1 part glacial acetic to 100 parts water). Casein was precipitated from the control solution, but not from the solution to which the powdered enzyme had been added, a result which shows that the enzyme can digest casein, at least to the stage where the products of its digestion are not precipitated in acid solution.

In order to test the action of this enzyme on casein in acid solution, small quantities of the powdered enzyme and the powdered casein were shaken in 5 c.c. of N/50 lactic acid. A control tube without the enzyme was also prepared. The two tubes were then placed in the incubator, and shaken at frequent intervals. In about 24 hours the casein enzyme tube became clear, while the casein in the control tube showed no evidence of digestion. In this respect also, this enzyme is unlike trypsin, which can digest casein only in approximately neutral or alkaline solutions.

*Fibrin*.—Finely divided fragments of fibrin were shaken up with some of the powdered enzyme in N/50 lactic-acid and N/50 sodium-hydroxid solutions. These, together with controls, were incubated at 37 C., with frequent shaking. In the tube containing the enzyme in alkaline solution digestion of the fibrin particles was complete in 8 hours; in that containing the acid solution, in 24 hours. The control tubes showed no digestion.

*Blood Corpuscles*.—A 2% suspension of washed blood corpuscles of the dog was divided into two 5-c.c. portions. Some of the powdered enzyme was inactivated by heating in the autoclave at 120 C. for 30 minutes, and then mixed with one of the suspensions for a control. The other suspension was mixed with an equal amount of the unheated enzyme, and the two tubes kept at 37 C. After 2 hours hemolysis was complete in the suspension containing the unheated enzyme, while the control suspension showed a clear supernatant fluid. Subsequent examination of the hemolyzed suspension with the microscope showed that the stroma of the cells had been digested. It appears from these experiments that the proteolytic enzyme of *B. proteus*, elaborated in the relatively simple medium of  $\text{NH}_4\text{Cl}$  peptone, has a digestive action on sub-

stances representing a rather wide range of protein characteristics, and for this reason should be considered a true protease rather than a "glease," as it has often been termed.

#### THE EFFECT OF HEAT ON THE STABILITY OF THE PROTEOLYTIC ENZYME OF *B. PROTEUS*

About 50 c.c. of a 48-hour  $\text{NH}_4\text{Cl}$ -peptone culture were boiled in a beaker over a flame, 1-c.c. portions being removed at intervals of 1, 5, 10, and 30 minutes, and mixed with 5 c.c. of carbol-gelatin (0.5% phenol in 15% gelatin). Other portions of the culture were heated in the autoclave for periods of 5 and 15 minutes at 120 C., and 1 c.c. of each portion was mixed with 5 c.c. carbol-gelatin. For controls, a tube containing 5 c.c. of gelatin with 1 c.c. of unheated culture and another with 1 c.c. of water were prepared. After 24 hours' incubation at 37 C. the tubes were removed from the incubator and allowed to cool to room temperature. The tube to which only water had been added, and the tube containing the portion of enzyme which had been heated in the autoclave at 120 C. for 15 minutes solidified at about the same time; next, the tube containing the enzyme which had been heated for 5 minutes at 120 C.; then the tube containing the enzyme of the 30 minutes' boiling; and last, after about 6 hours, the tube containing the enzyme of the 10-minutes' boiling. None of the other tubes became solid, even when placed in the ice-box, a fact showing that boiling for 5 minutes has no noticeable effect on the gelatin-digesting power of this enzyme. In this respect, again, it is unlike trypsin, which is destroyed before the boiling point is reached.

The method of heating was used also in testing the stability of the casein- and fibrin-digesting property of the enzyme of *B. proteus*. It was found that only after heating at 120 C. for 15 minutes was complete destruction obtained.

In using this method of determining the activity of the enzyme on gelatin, it is necessary that the tubes containing the gelatin be of the same diameter and thickness of glass, and in some cases it is even well to check up their order of solidifying by exchanging their contents.

#### THE DIFFUSIBILITY OF THE PROTEOLYTIC ENZYME OF *B. PROTEUS*

Two test tubes were suspended with their closed ends downward, about 1 cm. apart, within a beaker, the closed ends reaching to within 1 cm. of the bottom. Melted carbol-agar was then poured around them and allowed to solidify. The tubes were then carefully loosened and withdrawn from the agar, leaving 2 holes about 5 cm. deep. Carbol salt solution was poured into each hole and a small quantity of the powdered enzyme stirred into one of the holes. The salt solution in the other hole, after 24 hours, was able to digest gelatin, casein, and fibrin, proving thereby that the proteolytic enzyme of *B. proteus* still retains its activity on various proteins after diffusion through 1 cm. of solidified (2%) agar.

#### IS THE PROTEOLYTIC ENZYME OF *B. PROTEUS* A UNIT OR A MIXTURE OF ENZYMES?

From the foregoing experiments and others to be described later, it appears that the action of heat on the enzyme affects the ability of the enzyme to digest gelatin, casein, and fibrin to about the same degree

in each case. Similarly, the dialyzed product can act on all three of these proteins. Strongly acid reactions (N/20 lactic) retard, and alkaline reactions (N/50 to N/20 NaOH) accelerate the digestion of all three. Casein, fibrin, and gelatin are digested more quickly with a given quantity of  $\text{NH}_4\text{Cl}$ -peptone culture than with  $\text{NaCl}$ -peptone culture, while none of them is digested by  $\text{NH}_4\text{Cl}$ -peptone cultures containing 0.7% of calcium lactate. In other words, acids, alkalies,  $\text{NH}_4\text{Cl}$ , calcium lactate, heating, and dialyzing seem to affect the ability of the enzyme to digest these various proteins to the same degree. Such facts indicate that the enzyme is probably a unit, not a mixture of enzymes. The unity of trypsin is disputed by Pollak<sup>9</sup> because of its ability to digest gelatin in media of slightly acid reaction. Thus far it is seen that the only essential property which the enzyme of *B. proteus* has in common with trypsin is its ability to digest proteins of widely different natures. Other greater differences are presented in the experiments that follow.

## II

### THE AMOUNT OF ACID PRODUCED BY *B. PROTEUS* IN $\text{NH}_4\text{Cl}$ -PEPTONE MEDIA CONTAINING DIFFERENT AMOUNTS OF GLUCOSE

One-hundred-cubic-centimeter flasks of  $\text{NH}_4\text{Cl}$ -peptone media containing 0, 0.1, 0.2, 0.3, 0.4, and 1% of glucose were inoculated with *B. proteus* and placed in the incubator; 10-c.c. portions were removed every 24 hours and titrated with N/10 NaOH to determine the rate of increase of acid. Also, 1-c.c. portions were removed every 12 hours and mixed with 5 c.c. carbol-gelatin to determine the time of the appearance of the proteolytic enzyme.

*Results.*—(a) The flask containing no sugar showed no appreciable change in reaction after the first 24 hours. The enzyme was present at that time.

(b) The flask containing 0.1% glucose showed an acid reaction of approximately N/180 acid to litmus after 24 hours, and also a slight digestive action on gelatin.

(c) The 0.2% glucose flask showed its maximal acidity of approximately N/100 after 28 hours. The enzyme was not present until the 3rd day.

(d) The 0.3% glucose flask showed a reaction of N/70 after 72 hours, but the enzyme did not appear until the 5th day.

(e) The 0.4, 0.5, and 1% glucose flasks showed approximately equal amounts of acid (N/50) after 3 days, but no enzyme appeared in these flasks, even after 14 days.

<sup>9</sup> Hofmeister's Beiträge, 1904-5, 6, p. 95.

From the results obtained with the 0.1, 0.2, and 0.3% glucose flasks, it is seen that the proteolytic enzyme can be elaborated in media of a decidedly acid reaction, and that the order of appearance of the enzymes in these flasks is in the reverse order of the amounts of sugar present; that is, the enzyme appears first in the flasks containing the smaller amounts of glucose. From the results obtained with the 0.4, 0.5, and 1% glucose flasks, it appears that even tho an excess of sugar is present, *B. proteus* does not continue its action on carbohydrates in acid reactions above approximately N/50 acid to litmus. The reason for this will be shown in a subsequent experiment.

THE TIME ELAPSING BETWEEN THE DISAPPEARANCE OF A UTILIZABLE SUGAR FROM THE CULTURE AND THE APPEARANCE OF THE PROTEOLYTIC ENZYME

Flasks containing 200 c.c. of  $\text{NH}_4\text{Cl}$ -peptone medium to which 0.2% glucose had been added were inoculated with *B. proteus* and placed in the incubator. The rate of disappearance of the sugar was followed by removing 10-c.c. portions of the culture at regular intervals, and titrating by Bang's method of sugar-estimation. The sample of peptone used in this experiment gave a positive Molisch reaction even after 2 weeks' incubation with *B. proteus*. Altho this protein has a reducing action on the copper solution, the amount of reduction it causes was assumed to occur as a constant factor in the titration of each sample, since it apparently does not diminish in quantity in cultures of *B. proteus*.

TABLE 1  
AN AVERAGE OF THE RECORDS OF OBSERVATION ON CULTURES OF *B. PROTEUS*

Sample	Hours After Incubation	Amount in c.c. of Hydroxylamin Solution	Percentage of Dextrose*	Liquefaction of Gelatin	Amount in c.c. of N/10 NaOH Required to Make 10 c.c. of Culture Neutral to Litmus
1	Initial titration	29.0	.196	—	0.0
2	8	30.0	.186	—	0.2
3	24	36.5	.119	—	0.6
4	32	46.5	.03	—	1.1
5	48	50.0	0	+	1.0

\* Hammarsten: Text-Book on Physiological Chemistry, 1911, p. 765.

The strain of *B. proteus* used in these experiments was able to remove 0.2% glucose from these cultures in from 32 to 40 hours. By removing 1-c.c. portions at regular intervals and mixing with 5 c.c. carbol-gelatin it was found that the enzyme in no case appeared before the sugar was entirely removed from the culture, and usually appeared from 10 to 12 hours afterward.

It was thought that the appearance of the enzyme could be further delayed by using a larger percentage of sugar, and adding  $\text{CaCO}_3$  to the media to neutralize the acid as formed. Accordingly, by following the rate of the disappearance of the sugar in cultures containing 0.5% dextrose and 0.5%  $\text{CaCO}_3$  it was found that the entire quantity of sugar had disappeared on the 4th day after incubation. The appearance of the enzyme in this culture was tested by mixing 1-c.c. quantities of it with 5 c.c. carbol-gelatin, as in the case of the 0.2% dextrose culture already described. This was done daily for 14 days, but no formation of the enzyme was detected. The failure of the enzyme to appear in the  $\text{CaCO}_3$  media after the sugar had disappeared, recalled the statement made by Hirschler<sup>10</sup> that calcium lactate prevented the formation of indol, phenol, etc., a fact which he interpreted to mean that this substance is used for fuel purposes (the lactate ion being reduced to the propionate ion) and therefore has an action similar to that of the carbohydrates.

Inasmuch as calcium lactate is formed in such a culture as described—by the interaction of lactic acid and  $\text{CaCO}_3$ —the experiment of determining whether or not the presence of calcium lactate affects the metabolism of *B. proteus* in sugar-free and sugar-containing media suggested itself.

#### EFFECT OF LACTATE SALTS ON THE METABOLISM OF *B. PROTEUS*

(a) Fermentation tubes were filled with a medium containing 0.5%  $\text{NH}_4\text{Cl}$ , 0.5% glucose, and 1% peptone. To these tubes were added 0.1%, 0.2%, and so on up to 1% calcium lactate, and the series inoculated with *B. proteus*. After a 24-hour incubation, gas- and acid-production were almost completely inhibited in media containing as much as 0.7% of calcium lactate. The experiment was repeated with the ammonium and sodium salts of lactic acid. These were also found to have an inhibiting effect on the gas- and acid-formation.

The absence of any appreciable change in the reaction of these cultures is evidence that the lactic acid salts not only do not serve the fuel requirements of *B. proteus*, but also prevent in some way its normal utilization of the sugars. Enzymic actions in general are retarded, or even suspended entirely, by the presence of the products of reactions caused by a given enzyme, and this fact may serve to explain why the presence of these lactate ions inhibits the action of the endo-enzymes of *B. proteus*.

<sup>10</sup> Ztschr. f. physiol. Chem., 1886, 10, p. 306.

It will be shown in a subsequent experiment that the other ion of the lactic-acid molecule, namely, the hydrogen ion, has a similar effect—retarding and finally completely suspending the metabolic activities of endo-enzymes of *B. proteus*.

(b) Five tubes containing 10 c.c.  $\text{NH}_4\text{Cl}$ -peptone medium, and 5 tubes of the same medium to which 0.7% calcium lactate had been added, were inoculated with *B. proteus* and incubated. After 5 days the ammonia-production, as determined by the Folin air-current method, was approximately 5 times as great in the cultures which did not contain calcium lactate as in those which did, the result showing that this salt in some way interferes with the utilization of protein for fuel purposes. Furthermore, it also prevents the formation of the proteolytic enzyme, even after a 14-day incubation—the same observation which was made in the experiment described on p. 40. Indol was also absent in the cultures containing lactic-acid salts, as was observed by Hirschler in 1886. Macroscopic observation of the cultures, however, revealed no appreciable difference in the amount of growth in the two sets of tubes.

#### WHY IS THE PROTEOLYTIC ENZYME OF *B. PROTEUS* NOT FORMED IN CULTURES CONTAINING GLUCOSE?

The work of Kendall, Day, and Walker<sup>11</sup> gives two reasons why bacteria show a preference for utilizable sugars in ordinary sugar-protein media. In the first place, the protein molecule requires a digestion to simpler fragments before diffusion into the interior of the cell can occur, and since the oxidation of energy-giving reactions is brought about by the endo-enzymes of bacteria, these protein molecules can not be used for fuel as long as they can not diffuse into the interior of the cell. Sugar, on the other hand, readily diffuses into the cell body where it can be acted on by the zymase or endo-enzyme. In the second place, even tho protein in the digested form is added to sugar-protein media, its fragments, such as the amino-acids, etc., are more difficult to oxidize than are the utilizable sugars. In other words, the difference in the ability of these two foods, protein and sugar, to pass through the cell wall of the bacteria, and the difference in the readiness with which they are oxidized, are the two reasons why bacteria show a preference for sugar in protein-sugar media.

Unless an excess of sugar is present, this preference for sugar first causes the culture to become progressively acid in reaction as long as the sugar lasts. When the supply of the latter is exhausted, the bacteria are forced to change from a carbohydrate to a protein diet for fuel purposes. The products of protein-decomposition, such as indol, skatol, etc., then appear in the culture. Ammonia, being one of the

<sup>11</sup> Jour. Med. Research, 1911, 25, p. 155.

decomposition products resulting from a utilization of the proteins, causes a gradual decrease in the acid reaction until the culture may eventually become alkaline.

On the other hand, if more sugar is present than the bacteria can remove, the culture eventually reaches a condition, the nature of which is demonstrated in the following experiment.

A flask of 100 c.c. of  $\text{NH}_4\text{Cl}$ -peptone medium containing 1% glucose was inoculated with *B. proteus* and incubated at 37 C. until fermentation ceased, or for about 3 days. A portion of the culture was then filtered through a Berkefeld filter, and the bacteria-free filtrate removed with a sterile pipet to 2 sterile fermentation tubes. These two tubes were inoculated with a portion of the original 3-day-old culture. The bacteria used for the inoculation were then back in the medium from which they had been filtered—a medium which apparently contained something inhibiting the action of their sugar-fermenting enzyme. One of the tubes was neutralized with sterile N/10 NaOH, and the two tubes placed in the incubator. After 24 hours the tube which had been neutralized, showed the usual amount of growth, of gas, and of acid-formation, while the medium in the other tube, even after 2 weeks' incubation, remained perfectly clear and showed no gas-formation.

Since both growth and sugar-decomposition—that is, anabolism and catabolism—had occurred in the tube which had been neutralized, and were both absent in the other tube, it is evident that the bacteria had produced something which had suspended both functions of their metabolism—the building up and the tearing down of organic substances. Titratable acid in  $\text{NH}_4\text{Cl}$ -peptone media in concentration of N/50 acid to litmus seems to paralyze completely the action of the endo-enzyme of these bacteria, so that, altho an excess of energy-giving substances is present, the bacteria cannot appropriate them. In other words, when their endo-enzymes become paralyzed by certain products of their own reaction, the bacteria are virtually starved into a dormant condition.

### III

#### THE LIMITS OF ALKALINITY AND ACIDITY IN WHICH THE PROTEOLYTIC ENZYME OF *B. PROTEUS* CAN LIQUEFY GELATIN

In the previous experiments it was shown that titratable acid in broth in concentration of N/50 acid to litmus practically suspends the vegetative and reproductive functions of *B. proteus*. Since the bacteria cannot even multiply in this concentration of acid, the conclusion is warranted that this acidity (N/50) is the limit of the acid reaction in broth in which the organisms can elaborate their protein-digesting enzyme. It does not necessarily follow, however, that this is also the

limit of acidity which would still permit the action of this enzyme after it had been formed. In other words, the limit of acidity of the culture which still permits the formation of the enzyme, and that which still permits the activity of the enzyme after it is formed, are not necessarily the same, because the proteolytic enzyme is an extra-cellular product, and is not so closely associated with the life of the cell itself, as is the intracellular, sugar-decomposing enzyme. The following experiment, therefore, was undertaken to determine the stability of the enzyme with reference to acids and alkalies.

Carbol-gelatin of N/20, N/30, and N/40 titratable alkali (NaOH), and of N/20, N/30, and N/40 titratable acid (lactic) was prepared, and 5 c.c. of each of these placed in each of two tubes. With the content of one tube of each pair was mixed 1 c.c. of a 48-hour  $\text{NH}_4\text{Cl}$ -peptone culture of *B. proteus*, and with that of the other one of each pair, 1 c.c. of water, for controls. After 18 hours at 37 C. the tubes were removed from the incubator and allowed to cool to room temperature. The controls without the enzyme, all solidified in approximately the same length of time (15 minutes), a result showing that no hydrolysis of the gelatin, due to the action of the acid or alkali, had taken place. Of those tubes containing the 1 c.c. of culture—that is, of the enzyme—none showed any tendency to become solid after standing 2 hours, a result showing that even in dilutions of 1:6, this enzyme is able to digest gelatin to the point of liquefaction in 18 hours, in media to which has been added titratable acid in concentrations of N/20 acid to litmus.

In previous experiments, it was demonstrated that *B. proteus* can not form this enzyme in 1% peptone in hydrogen-ion concentration greater than that corresponding to N/50 titratable acid. The immediately foregoing experiment, however, shows that much greater acid reactions than N/50 do not prevent the action of this enzyme after it is once formed. This may be considered as further evidence that the failure of *B. proteus* to liquefy sugar-gelatin cultures is not due to the acid reaction of the culture, because this acid reaction reaches a maximum of only approximately N/50, whereas *B. proteus* is known to be active in acid reaction of as much as N/20, and that, too, in dilution of 1 part of culture to 5 parts of gelatin.

It has been stated before that the active agent affecting the progress of changes produced by bacteria and their enzymes is the dissociated hydrogen and not the titratable acid. It should be noted here, however, that the 15%-gelatin-0.5%-carbol mixture used in this test, and the glucose- $\text{NH}_4\text{Cl}$ -peptone media used in Experiment II have approximately the same tendency to repress the dissociation of the hydrogen ion of lactic acid. The great difference, therefore, between the amount of titratable acid in the N/20-acid gelatin of this experiment and in

the N/50-acid broth of Experiment II(e) is sufficient evidence that a correspondingly great difference in the hydrogen-ion content of the two media also exists. Objection that the enzyme can not be elaborated in an acid reaction, or else is destroyed in some stage of its formation can hardly be made. Both Auerbach<sup>5</sup> and Hirschler<sup>7</sup> kept their cultures neutral with MgO and CaCO<sub>3</sub> and still found no protein-decomposition taking place in sugar-containing media. Furthermore, the 0.1, 0.2, and 0.3%-glucose flasks of Experiment II were all of distinctly acid reaction for several hours or days, and in spite of this the organisms were able to form the enzyme after the sugar had been exhausted.

It might be asserted that some by-product of the sugar-decomposition other than the hydrogen ion is responsible for the failure of sugar-gelatin cultures to become liquefied. To determine whether this is the case, the following experiments were performed:

(a) One 10-c.c. tube of sugar-free nutrient gelatin and 2 tubes of 1% glucose gelatin were inoculated with *B. proteus*. After 24 hours the sugar-free gelatin was liquefied. The two 1%-glucose-gelatin tubes were still solid, and remained so, even after 14 days. When the three tubes had been incubated for 14 days, 1 c.c. of the liquefied gelatin of the glucose-free culture (containing the enzyme), was transferred to one of the 1%-glucose-gelatin cultures and both glucose-gelatin cultures returned to the incubator. After 6 hours at 37 C. the sugar-gelatin tube to which the 1 c.c. of sugar-free gelatin had been added was liquefied, in spite of its acid reaction, while the other glucose-gelatin tube was still solid.

(b) This experiment was repeated with two 10-c.c. tubes of milk to which 1% of glucose was added, and one plain milk tube. After a 7-day incubation with *B. proteus* 1 c.c. of the plain culture (which was almost completely peptonized) was added to one of the 1% glucose-milk tubes. The two glucose-milk cultures had been coagulated by the acids formed from the glucose, but they showed no signs of peptonization. After 3 days in the incubator the glucose-milk tube, to which the 1 c.c. of plain-milk culture had been added, was well peptonized, while the other showed no such change. The undigested curd from the first tube weighed only 0.08 gm., while that from the other tube weighed 0.45 gm.

These experiments apparently justify the same conclusion; namely, that the absence of protein changes in sugar-containing media is not to be attributed to the inhibiting effect of hydrogen ion, or some other substance, or to the action of a trypsin-like ferment, but simply to the fact that the enzyme which makes possible the utilization of the proteins for fuel purposes does not appear in the culture as long as a utilizable carbohydrate is present.

## SUMMARY

The proteolytic enzyme of *Bacillus proteus* is insoluble in 98% alcohol. The dried enzyme is active after months of exposure to light and air, and is not completely destroyed by boiling for 30 minutes; it is diffusible through one-half inch of 2% agar; it acts best in alkaline solution, but can digest fibrin gelatin and casein in solutions of N/50 or more acid reaction. Failure to destroy its power to digest one protein while leaving intact its ability to digest another indicates that it is a unit rather than a mixture of enzymes.

*B. proteus* incubated in sugar-containing medium until it has produced its maximal acidity, refuses to grow when re-inoculated into this medium filtered. This failure to grow is probably to be attributed to the paralyzing effect of hydrogen ion on the endo-enzyme of the bacteria, since subsequent neutralization of the media restores the vegetative and reproductive functions of the bacteria. The vegetative function is also hindered by the presence of the lactate ion, either as sodium, ammonium, or calcium salts, but to a less degree than in the case of the hydrogen ion.

Nutrient gelatin to which has been added as much as 0.5% of a utilizable sugar becomes acid after a few hours' incubation with *B. proteus*, and the gelatin of the medium is not liquefied, even after weeks or months of incubation. The constant concurrence in cultures of certain bacteria of these two phenomena—the acidity of the cultures on the one hand, and the absence of the proteolytic changes in the gelatin, or other protein, on the other—seems to permit but two possible explanations. Either (1) the gelatin-liquefying enzyme of *B. proteus*, and of certain other bacteria, is trypsinic in nature and therefore unable to act in the presence of the acid of the cultures, or else (2) the enzyme is not present in cultures containing a utilizable carbohydrate. The fact that a small quantity of the enzyme, either as the dried powder or in culture form, when added to a sugar-gelatin culture that has reached its maximal acidity, causes liquefaction of this culture after 6 hours at 37 C., indicates that this enzyme acts readily in cultures of acid reaction, and that the second explanation is therefore the more plausible—namely, that the absence of the proteolytic changes in sugar-containing media is due to the fact that the enzyme causing these changes does not appear in cultures containing a utilizable carbohydrate.

# THE ANTIGENIC VALUE OF SPIROCHÆTA HYOS IN COMPLEMENT-FIXATION TESTS ON HOG- CHOLERA SERA \*

## STUDIES ON HOG CHOLERA

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No references to laboratory methods for the diagnosis of hog cholera occur in the earlier literature with the exception of that to the use of *Bacillus cholera-suis* in experimental agglutination tests.<sup>1</sup> Within the last few months reports have been made of the results of some experimental complement-fixation reactions.

Connaway<sup>2</sup> and his associates found that antigen prepared from the blood of pigs suffering from hog cholera was unsatisfactory. Negative results also followed the use of antigens prepared from the spleens and kidneys of virus pigs.

Healy and Smith<sup>3</sup> have published results obtained with an antigen prepared from the mesenteric glands of cholera hogs. This was made by grinding 18 gm. of selected mesenteric-gland tissue with sterile sand. To this, 180 gm. of neutral 1% glucose broth were added, and the mixture allowed to stand for 8 days at 4 C. The results of tests with this material led the authors to conclude that they had obtained "an antigen which shows striking differences in its reaction toward normal hog, rabbit, and cow sera, and hyperimmune hog serum. The antigen is not present in freshly prepared extract of mesenteric glands, but requires a definite period for development; it is not removed from such an extract by passage through an ordinary porcelain filter but is removed by passage through the F bougie. Finally it gradually disappears from the extract." These investigators state that they are seeking to perfect the preparation of the antigen which they have developed, with a view to rendering it more sensitive.

Our study of *Spirochaeta hyos*,<sup>4</sup> an organism present in the intestinal ulcers, cecal crypts, and external local lesions of animals suffering from hog cholera, led us to undertake a series of experiments to determine its antigenic value in complement-fixation, a project apparently justified by the results of a rather extensive investigation of this organism.

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<sup>1</sup> Giltner: Tech. Bull. Mich. State Exper. Sta., No. 8, 1911; No. 13, 1912.

<sup>2</sup> Bull. Missouri State Exper. Sta., 1914, 131, p. 486.

<sup>3</sup> Jour. Infect. Dis., 1915, 17, p. 213.

<sup>4</sup> King and Baeslack: Jour. Infect. Dis., 1913, 12, p. 307. King, Baeslack and Hoffman: Ibid., p. 365. King and Hoffman: Ibid., 13, p. 463. King and Drake: Ibid., 1914, 14, p. 246. King, Drake and Hoffman: Ztschr. f. Immunitätsf., 1914, 22, p. 347. King and Drake: Jour. Infect. Dis., 1915, 16, p. 1.

The complement-fixation test is recognized as one of the most reliable methods of laboratory diagnosis in specific infections such as syphilis, gonorrhea, glanders, contagious abortion, and dourine. The test is also of practical use in the standardization of certain antisera, as antimeningococcic and antigonococcic sera, and in checking up the specificity of pathogenic micro-organisms.

It is unnecessary to discuss in detail the methods which should be followed in routine complement-fixation work, but for the purpose of recording our results clearly and completely, the following explanations are given.

#### APPARATUS

The tests are carried out in small tubes, 50 mm. in length by 8 mm. in diameter. In addition to the ordinary pipets graduated to 10ths and 100ths, it is a convenience to have small pipets of 0.1-c.c. capacity, made especially for the work from thermometer glass and graduated by mercury into 100 parts, thus affording readings to 0.001 c.c. The solutions used are normal salt solution (0.85 NaCl to 100 c.c. distilled water) and sodium-citrate solution (1% sodium citrate in normal salt solution). Special care is exercised in cleaning tubes, pipets, and other glassware; if chromic-acid cleaning solution is used, the apparatus is very thoroughly rinsed, first in tap water and then in sterile water, before drying and sterilizing, to eliminate inaccuracies due to the presence of foreign matter.

#### REAGENTS

*Sheep Corpuscles.*—Fresh sheep blood is collected in sodium-citrate solution. The red cells are secured by repeated centrifugation and at least 4 washings in normal salt solution. Finally the cells are placed in normal salt solution in 1% suspension. This suspension may be kept for several days in the refrigerator.

*Amboceptor.*—The amboceptor, hemolytic serum, is obtained from a rabbit previously injected intravenously with varying doses of washed sheep corpuscles. The rabbit should receive injections of 4, 6, and 8 c.c. respectively, at intervals of 7 days. Ten days after the last injection, the rabbit is bled and the serum obtained.

*Complement.*—The complement, normal guinea-pig serum, is obtained each day in fresh condition and it should be clear.

*Antigen.*—Spirochetes are obtained by centrifugation from a pure liquid culture of *Spirochaeta hyos*. The sediment of pure *Spirochaeta hyos* thus obtained, is washed with normal salt solution, the supernatant liquid removed, and 20 times its volume of absolute alcohol added to the mass of washed spirochetes. The suspension is placed in a mechanical shaker for 24 hours, after which it is incubated at 37 C. for a period of 10 days, being shaken by hand a few times each day during this period. At the end of this time the suspension is diluted with an equal volume of normal salt solution. It is then ready for titration.

In the present work the strain of *Spirochaeta hyos* used in the preparation of antigen was secured from the intestinal ulcers of Hog 112. This animal was infected with a strain of hog-cholera virus received from Dr. Moore and Dr. Birch of Cornell University. The antigen was obtained from pure cultures of *Spirochaeta hyos* grown under oil in ascitic-broth media to which had been added sterile rabbit kidney or testicular tissue.

*Serum to be Tested.*—The serum to be tested is obtained under aseptic conditions free from red corpuscles and hemolysis. Before use, it is inactivated by heating to 56 C. for 30 minutes in a water bath.

#### TITRATION OF REAGENTS

*Amboceptor.*—The serum from the rabbit immunized against sheep corpuscles is inactivated by heating in a water bath at 56 C. for 30 minutes. Dilutions of the serum are then made, from 1:100 to 1:2500, and by titration of these with 0.01 c.c. of complement and 1 c.c. of the 1% suspension of washed sheep cells, that dilution is found in which complete hemolysis of the red cells occurs in 1 hour. This represents the amboceptor unit. Twice this amount is used in the test.

*Complement.*—The complement, fresh normal guinea-pig serum, is titrated against the amboceptor unit thus obtained, for the purpose of determining any variation in the complementary properties of the fresh guinea-pig sera. This titration is carried out each day before the test. The complementary unit is the smallest amount of complement that will completely hemolyze 1 c.c. of a 1% suspension of sheep cells in the presence of 1 unit of amboceptor. For example, 0.005 c.c. complement after 1 hour at 37.5 C. in the water bath, caused partial or no hemolysis; 0.01 c.c., 0.015 c.c., and 0.02 c.c. gave complete hemolysis while the control remained unhemolyzed. Twice the complementary unit is used in the test.

*Antigen.*—The antigen must be titrated for the presence of hemolysins. Tubes containing different amounts of antigen, 0.005, 0.01, 0.03, 0.05, and 0.1 c.c., respectively, and one containing no antigen, as control, together with 2 units of complement and 1 c.c. of the sheep-cell suspension, are incubated for 1 hour at 37.5 C. in a water bath. None of the tubes should show hemolysis. If all the tubes show hemolysis, either the complement or the cell suspension, or both, are hemolytic. If there is hemolysis in all the tubes except the control tube, the antigen itself is hemolytic and should be discarded.

The antigen in amounts of 0.005, 0.01, 0.02, 0.05, and 0.1 c.c., is titrated against 2 units of complement, 2 units of amboceptor, and 1 c.c. of sheep-cell suspension for the purpose of detecting the presence of any anticomplementary properties. A control tube containing no antigen is also prepared. If there is complete hemolysis in each case after incubation at 37.5 C. for 1 hour, the absence of anticomplementary properties is demonstrated. If hemolysis occurs in the control tube, and inhibition is present in any of the tubes containing the larger amounts of antigen, the amount of antigen used must be less than that causing any inhibition of hemolysis.

For the purpose of determining its antigenic properties the antigen is titrated against a known positive and a known normal, or negative, serum as illustrated in Table 1.

TABLE 1  
 THE TITRATION OF ANTIGEN FOR ITS ANTIGENIC PROPERTIES

Tube	Amount of Serum, c.c.	Units of Complement	Amount of Antigen,* c.c.
1	Cholera.....	2	0.005
2		2	0.01
3		2	0.015
4		2	0.02
5		2	0.0
6	Normal.....	2	0.005
7		2	0.01
8		2	0.015
9		2	0.02
10		2	0.0

\* The antigen, in the largest amount used, should have previously shown no anticomplementary properties.

The tubes are incubated in a water bath at 37.5 C. for 1 hour. Then to each tube are added 1 c.c. of sheep cells and 2 units of amboceptor and the tubes are again incubated in a water bath at 37.5 C. for 1 hour. At the end of the hour, after the sheep cells are added, there should be complete hemolysis in Tubes 5, 6, 7, 8, 9, and 10, but of the first 4 tubes, those containing sufficient antigen to bind the complement, should show complete inhibition of hemolysis. From this titration the amount of antigen necessary to cause fixation of complement is determined and used as the antigenic unit for the actual test.

### THE TEST

Table 2 will illustrate the method of conducting the complement-fixation test.

 TABLE 2  
 COMPLEMENT-FIXATION TEST IN HOG-CHOLERA

Tube	1	2	3	4*
Sera used (c.c.) { 217 (normal)..... 207 (known positive) Lapeer (unknown)...	0.02	0.04	0.06	0.06
Antigen (c.c.).....	0.01	0.01	0.01	0.0
Complement (c.c.).....	0.03	0.03	0.03	0.03
The tubes were incubated in a water bath for 1 hour at 37.5 C.				
Amboceptor (c.c.).....	0.04	0.04	0.04	0.04
Cells (c.c.).....	1.0	1.0	1.0	1.0
Hemolysis after 40 minutes:				
Serum 217.....	Complete	Complete	Complete	Complete (control)
Serum 207.....	++	—	—	Complete
Serum from Lapeer.....	++	—	—	++

\* Since antigen, complement, amboceptor, and cells had been previously tested for anticomplementary properties and hemolysis, the only control of the test was the serum control (Tube 4).

That there was some slight inhibiting action in the case of the Lapeer serum is shown by the failure of Tube 4 to hemolyze completely, but as no hemolysis had taken place in Tubes 2 and 3, a positive reading was given. Of the

tubes containing cholera serum (207), Nos. 2 and 3 showed no hemolysis and therefore positive results were recorded. Hemolysis occurred in all tubes containing Serum 217 (normal); negative results were recorded. It was shown that 0.02 c.c. of serum was insufficient in amount to cause complete complement-fixation—that is, to prevent partial hemolysis.

Results should be read when the action of the controls is complete. If the test shows complete hemolysis in the tubes (see Tubes 1, 2, and 3), it is evident that there has been no fixation of complement by the serum; therefore, the serum is negative (—). If there has been no hemolysis, the complement is bound and the serum is positive (++++). If only a slight degree of hemolysis has taken place, the result is recorded as triple plus (+++). When only about one-half of the cells have hemolyzed, the reading is given double plus (++) , while if there are only a few cells left unhemolyzed but still easily seen, a reading of one plus (+) is made. A one plus (+) is interpreted as doubtful; double plus (++) , triple (+++) , and four plus (++++) as positive.

The readings should be checked up after the tubes have been allowed to stand for several hours; the tubes containing known normal serum should show complete hemolysis, while those representing positive, or cholera, serum, should remain unhemolyzed. The action frequently occurs in less than 1 hour after the cells are added, and in such cases rapid hemolysis may be partially checked by placing the tubes in an incubator or at room temperature instead of in the water bath.

Known positive and negative sera must be subjected to test with the unknown sera to insure proper titration of all reagents. It is obvious that the amounts of sera used should be varied, as, for example, in the test described, in which the smaller amount of serum was found insufficient to cause complete complement-fixation. The amounts of sera used should not be too large on account of the inhibition of hemolysis which might result. This is controlled by Tube 4. Occasionally a serum is found which possesses inhibitory properties. Such a serum must be titrated carefully to determine the amount in which inhibition is negligible.

#### THE STRAINS OF VIRUS UTILIZED

Five different strains of hog-cholera virus have been used in conducting these experimental complement-fixation tests. Strain 1 (N. Y.) was received from Dr. Moore and Dr. Birch, of Cornell University. Strain 2 (from Dr. Hauk, of East St. Louis) represented a stock strain built up by mixing together all the strains of virus obtainable. Some of the original strains incorporated in this were secured from field cases, some from the government laboratories at Ames, Iowa, and some from serum-manufacturing laboratories. Dr. Hadley and Dr. Beach, of the University of Wisconsin, furnished Strain 3 (Wisconsin). Strain 4 was secured on October 21 from a cholera-infected herd of hogs at Grosse Isle, Mich. During the week of October 25, hog cholera appeared on the farm of the Michigan state school for feebleminded children, at Lapeer. One test was conducted with a specimen of serum obtained during this outbreak (Strain 5, Lapeer).

Serum was tested from one animal, Hog 63, infected with Strain 6 (Eloise). Hog 63 had received impure cultures of *Spirochaeta hyos* isolated from the intestinal ulcers in pigs that had received virus from an outbreak of hog cholera on the farm of the Wayne county hospital, Eloise, Mich.

The following hogs, infected with the different strains of virus, were used in the complement-fixation tests:

Strain 1 New York	Strain 2 St. Louis	Strain 3 Wisconsin	Strain 4 Grosse Isle	Strain 5 Lapeer	Strain 6 Eloise	Unknown Strains
77	187	188	206	Strain from case in field	63	192
160	161	203	223			189
186	202	217	227			220
106	141	205				
87	207	216				
204	224	218				
208	229	222				
221		214				
230		228				
		215				

## SUMMARY OF GENERAL DATA

With antigen prepared from pure cultures of *Spirochaeta hyos* there have been conducted 115 complement-fixation tests. Of these, 22 were with normal hog sera from 10 different animals, 1 with serum from an animal which exhibited a reaction only, following inoculation with virus, 6 with sera from 2 convalescent or naturally immune swine, 84 with sera from 34 animals suffering from hog cholera (4 of which had been used as normals), and 1 test each with 2 different lots of hyper-immune serum. Table 3 shows the results obtained.

TABLE 3

COMPLEMENT-FIXATION TESTS WITH ANTIGEN FROM PURE CULTURES OF SPIROCHAETA HYOS

Test	Date of Test	Animal	Date of Collection of Serum	Clinical Condition of Animal	Number of Days After Inoculation	Result of Complement Fixation Test	Remarks
1	10/ 5	Normal A	10/ 5	Normal	..	—	
2	10/ 5	77	3/23	Hog cholera	10	++++	Autopsy on 10th day
3	10/ 7	77	3/23	Hog cholera	10	+++	
4	10/ 5	160	8/18	Hog cholera	9	++	Autopsy on 9th day
5	10/22	160	8/18	Hog cholera	9	++	Autopsy on 9th day
6	10/ 7	Normal B	10/ 7	Normal	..	—	
7	10/ 8	Normal B	10/ 7	Normal	..	—	
8	10/ 8	186	10/ 8	Hog cholera	10	—	Error in technic. See Test 9
9	10/13	186	10/ 8	Hog cholera	10	++++	Autopsy on 16th day
10	10/ 8	187	10/ 4	Hog cholera	17	+++	Autopsy on 18th day
11	10/ 8	188	10/ 3	Hog cholera	12	+++	Autopsy on 12th day
12	10/13	Normal C	10/13	Normal	..	—	
13	10/26	Normal C	10/13	Normal	..	—	
14	10/13	106	5/ 6	Hog cholera	10	++++	Autopsy on 10th day
15	10/13	161	8/23	Hog cholera	13	++++	Autopsy on 13th day
16	10/22	161	8/23	Hog cholera	..	++++	
17	10/26	161	8/23	Hog cholera	..	++++	
18	11/ 4	161	8/23	Hog cholera	..	++	
19	10/21	Normal D	10/20	Normal	..	—	
20	10/22	Normal D	10/20	Normal	..	—	

TABLE 3—Continued

COMPLEMENT-FIXATION TESTS WITH ANTIGEN FROM PURE CULTURES OF SPIROCHAETA HYOS

Test	Date of Test	Animal	Date of Collection of Serum	Clinical Condition of Animal	Number of Days After Inoculation	Result of Complement Fixation Test	Remarks
21	10/21	192	10/20	Hog cholera	13	—	Error in technique. See Test 23
22	10/22	192	10/20	Hog cholera	13	±	Error in technique. See Test 23
23	10/26	192	10/20	Hog cholera	13	++++	Autopsy on 19th day
24	10/21	Hyperimmune serum	.....	.....	..	+++	Received from Michigan exper. station
25	10/21	87	4/15	Hog cholera	10	++++	Autopsy on 10th day
26	10/21	63	1/29	Hog cholera	16	+++	
27	10/29	63	1/29	Hog cholera	16	++++	Autopsy on 16th day
28	10/22	203	10/22	Hog cholera	10	+	See Test 29
29	10/26	203	10/22	Hog cholera	10	++++	Died on 17th day
30	10/22	202	10/22	Hog cholera	10	+	Died on 31st day
31	10/22	Grosse Isle	10/21	Hog cholera	..	+	Secured in field from moribund animal. See Test 32
32	10/26	Grosse Isle	10/21	Hog cholera	..	+++	Secured in field from moribund animal
33	10/29	217	10/29	Normal	..	—	
34	11/ 2	217	10/29	Normal	..	—	
35	11/12	217	11/12	Hog cholera	8	++	Autopsy on 13th day
36	10/29	204	10/27	Symptoms, Temp. 106 F.	9	++	Animal had reaction only
37	11/ 2	204	11/ 1	No symptoms	14	+	Became normal on 13th day
38	11/11	204	11/10	Normal immune	23	—	Exposed with Hogs 208 and 221
39	11/29	204	11/22	Normal immune	34	—	
40	12/ 2	204	11/30	Normal immune	42	—	
41	10/29	Hyperimmune serum	.....	.....	..	+++	Received from Dr. Huff, Sioux City, Iowa
42	10/29	141	9/11	Hog cholera	14	++++	Autopsy on 14th day
43	11/ 2	141	9/11	Hog cholera	..	++++	
44	11/ 2	205	11/ 1	Hog cholera	4	++	Temp. 105. Clinical symptoms 5th day
45	11/10	205	11/ 9	Hog cholera	12	+++	
46	11/11	205	11/ 9	Hog cholera	12	++	
47	11/19	205	11/17	Hog cholera	20	++++	Found dead on 23rd day
48	11/ 2	206	11/ 1	Natural immune	4	—	
49	11/ 2	207	11/ 1	Hog cholera	4	+++	Temp. 105.8. Clinical symptoms. Found dead on 19th day
50	11/ 2	Lapeer serum	10/30	Hog cholera	..	++++	Natural exposure in field
51	11/ 9	216	11/ 3	Normal	..	—	
52	11/10	216	11/ 3	Normal	..	—	
53	11/12	216	11/12	Hog cholera	8	+++	Autopsy on 13th day
54	11/ 4	220	11/ 3	Hog cholera	?	++++	Accidental exposure
55	11/11	220	11/ 3	Hog cholera	?	+++	Found dead on 13th day
56	11/ 4	208	11/ 3	Hog cholera	6	++++	Symptoms
57	11/10	208	11/ 9	Hog cholera	12	++	
58	11/15	208	11/10	Hog cholera	13	++++	
59	11/15	208	11/ 9	Hog cholera	12	++++	
60	11/16	208	11/ 9	Hog cholera	12	++++	
61	11/29	208	11/24	Hog cholera	27	+++	Moribund
62	11/ 4	189	11/ 4	Hog cholera	?	+++	Accidental exposure. Found dead 11/12
63	11/10	Normal E	11/ 9	Normal	..	—	
64	11/11	Normal E	11/ 9	Normal	..	—	
65	11/18	Normal E	11/ 9	Normal	..	—	

TABLE 3—Continued

COMPLEMENT-FIXATION TESTS WITH ANTIGEN FROM PURE CULTURES OF SPIROCHAETA HYOS

Test	Date of Test	Animal	Date of Collection of Serum	Clinical Condition of Animal	Number of Days After Inoculation	Result of Complement-Fixation Test	Remarks
66	11/11	218	11/10	Hog cholera	6	+	Autopsy on 13th day
67	11/12	221	11/11	Normal	..	—	
68	11/12	221	11/12	Hog cholera	1	—	No symptoms, no fever
69	11/15	221	11/12	Hog cholera	1	—	No symptoms, no fever
70	11/29	221	11/12	Hog cholera	1	—	
71	11/15	221	11/13	Hog cholera	2	—	
72	11/16	221	11/13	Hog cholera	2	—	
73	12/ 1	221	11/13	Hog cholera	2	—	No symptoms, no fever
74	11/16	221	11/14	Hog cholera	3	—	
75	11/16	221	11/15	Hog cholera	4	++	
76	11/18	221	11/15	Hog cholera	4	+++	
77	11/16	221	11/16	Hog cholera	5	+	Slightly inactive, no fever
78	11/19	221	11/16	Hog cholera	5	+++	
79	11/18	221	11/17	Hog cholera	6	+++	Temp. 104.2
80	11/18	221	11/18	Hog cholera	7	—	Error in technic. See Test 81
81	11/19	221	11/18	Hog cholera	7	++++	Temp. 106. Symptoms
82	11/19	221	11/19	Hog cholera	8	++++	
83	11/29	221	11/22	Hog cholera	11	+++	
84	12/ 2	221	11/24	Hog cholera	13	++++	
85	12/ 7	221	11/29	Hog cholera	18	++++	Animal died on 20th day
86	11/15	222	11/11	Normal	..	—	
87	11/12	222	11/12	Hog cholera	1	—	
88	11/15	222	11/12	Hog cholera	1	—	
89	11/15	222	11/13	Hog cholera	2	—	
90	11/16	222	11/14	Hog cholera	3	+++	Temp. 106.2. No clinical symptoms
91	11/16	222	11/15	Hog cholera	4	+++	
92	11/16	222	11/16	Hog cholera	5	+++	Clinical symptoms pronounced
93	11/18	222	11/17	Hog cholera	6	+++	
94	11/18	222	11/18	Hog cholera	7	+	See Test 95
95	11/19	222	11/18	Hog cholera	7	++++	
96	11/19	222	11/19	Hog cholera	8	++++	Found dead on 10th day
97	11/15	Normal F	11/15	Normal	..	—	
98	11/19	Normal F	11/15	Normal	..	—	
99	11/18	223	11/17	Hog cholera	6	—	Field virus, Grosse Isle. Temp. 105.4. Clinical symptoms
100	11/29	223	11/22	Hog cholera	11	+++	Found dead on 21st day
101	11/29	214	11/22	Hog cholera	11	+++	
102	12/ 1	214	11/22	Hog cholera	11	+++	
103	12/ 2	214	11/22	Hog cholera	11	+++	Found dead on 18th day
104	11/29	215	11/19	Chronic hog cholera	8	—	No symptoms. Temp. 104.2
105	12/ 2	215	11/29	Chronic hog cholera	18	+++	Marked symptoms
106	11/29	224	11/19	Hog cholera	8	+++	Autopsy on 25th day
107	12/ 7	227	12/ 2	Hog cholera	5	+	Temp. 104. Slight symptoms
108	12/ 7	227	12/ 4	Hog cholera	7	+++	Temp. 106. Marked symptoms
109	12/ 7	228	12/ 2	Hog cholera	5	+	Temp. 106. Symptoms
110	12/ 7	228	12/ 4	Hog cholera	7	++++	Temp. 107. Symptoms
111	12/ 7	Normal G	12/ 6	Normal	..	—	
112	12/ 7	229	12/ 2	Hog cholera	5	+	No symptoms. Temp. 103.6
113	12/ 7	229	12/ 4	Hog cholera	7	++	No symptoms. Temp. 104.8
114	12/ 7	230	12/ 2	Hog cholera	5	+	No symptoms. Temp. 104
115	12/ 7	230	12/ 4	Hog cholera	7	+++	No symptoms. Temp. 105.4

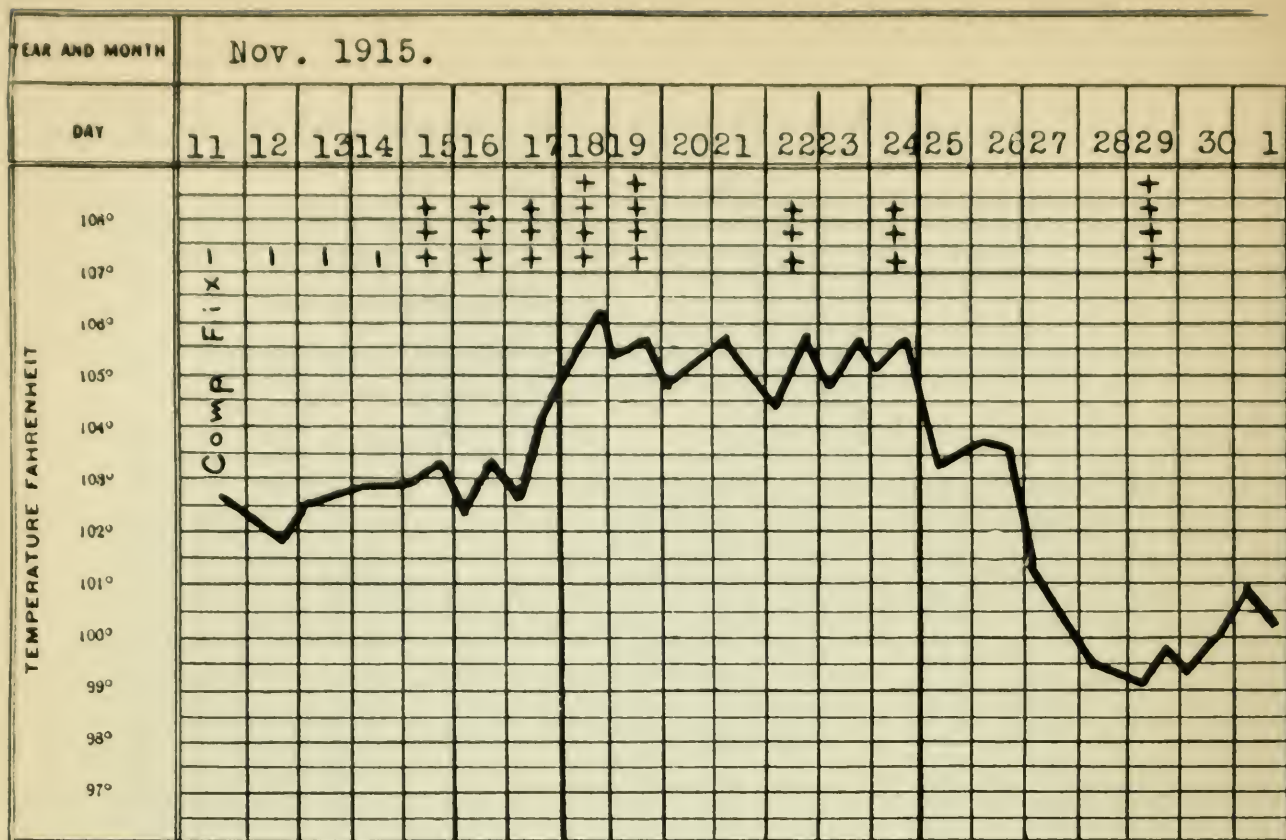


Chart 1. Clinical chart of Hog 221, showing also the time of the appearance of complement-fixation. November 11, intramuscular injection of 2 c.c. of Virus 208, Strain 1 (N. Y.). November 16, slight symptoms. November 18, marked symptoms. November 22, acute hog cholera. December 2, found dead. Examined.

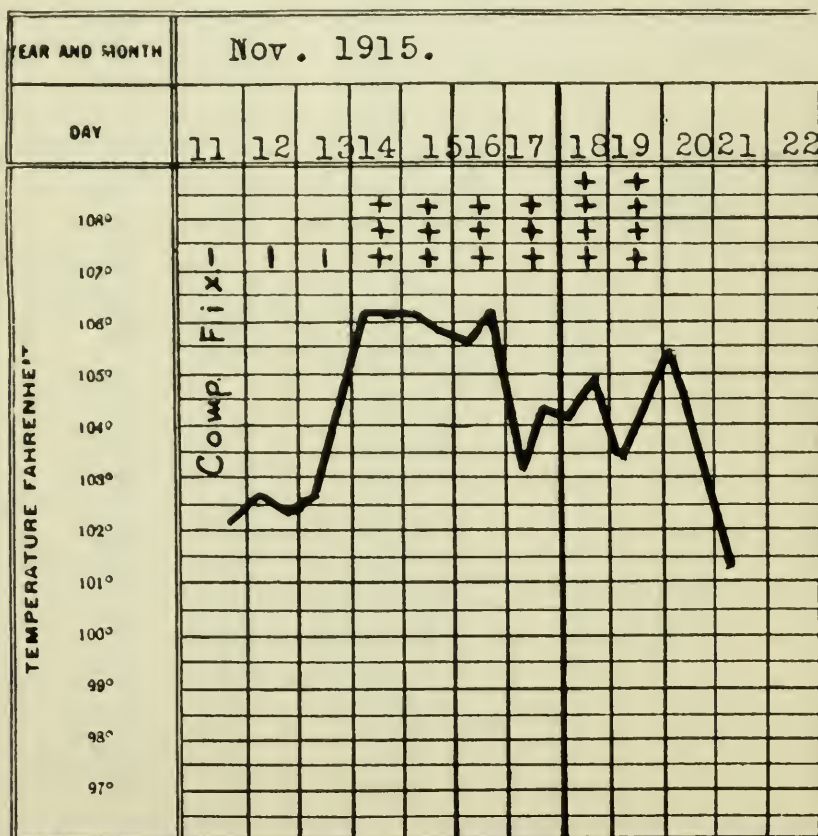


Chart 2. Clinical chart of Hog 222, showing also the time of the appearance of complement-fixation. November 11, intramuscular injection of 2 c.c. of Virus 205, Strain 3 (Wis.). November 15, slight symptoms. November 16, marked symptoms. November 17, acute hog cholera. November 22, found dead. Examined.

These results may be summarized as follows: (1) Hemolysis (—) occurred in all cases in which normal hog sera were subjected to complement-fixation test. (2) Complement-fixation (+) resulted in all tests with sera from cholera hogs, except in Nos. 30 and 66

#### THE TIME OF THE APPEARANCE OF A POSITIVE REACTION

In order to determine the number of days after inoculation before complement-fixation appears, daily examinations were made of the sera of two experimentally infected animals (221 and 222). The results obtained are shown in Charts 1 and 2.

With the sera of Hogs 222 and 221, positive serum reactions occurred in 3 and 4 days, respectively. These results corresponded approximately with the apparent periods of incubation, variation in resistance, and types of the disease present in these animals. Hog 222 exhibited a temperature of 106.2 on the morning of the 3rd day, clinical symptoms on the 4th day, and died on the 10th day. Hog 221, the serum of which gave a positive reaction one day later than that of Hog 222, did not show clinical symptoms until the 5th day, or rise of temperature until the 6th day, and lived until the 20th day.

Additional data bearing on this point are presented in Table 4.

TABLE 4  
THE TIME OF THE APPEARANCE OF COMPLEMENT-FIXATION

Hog	Incubation Period According to Temperature and Clinical Conditions	Duration of Disease in Days	Type of Disease	Complement-Fixation Test		Results of Subsequent Complement-Fixation Tests
				Day After Inoculation	Result	
205	4 days	23	Subacute	4th	++	12th day, +++; 20th day, +++++
207	4 days	19	Subacute	4th	+++	
227	4 days	..	Acute	5th	+	7th day, +++
228	4 days	..	Acute	5th	+	7th day, +++++
229	7 days	..	Subacute	5th	+	7th day, ++
230	7 days	..	Subacute	5th	+	7th day, +++
208	5 days	27	Chronic	6th	++++	12th and 13th days, ++++; 27th day, +++
218	4 days	13	Acute	6th	+	
223	6 days	21	Subacute	6th	—	11th day, +++
215	9 days	31	Chronic	8th	—	18th day, +++

The results of serum tests applied before symptoms appear or early in the course of the disease, indicate that complement-fixation is coincident with clinical symptoms, and that the time of its appearance depends on the virulence of the infecting material and the individual resistance of the animal.

Oct. 1915.

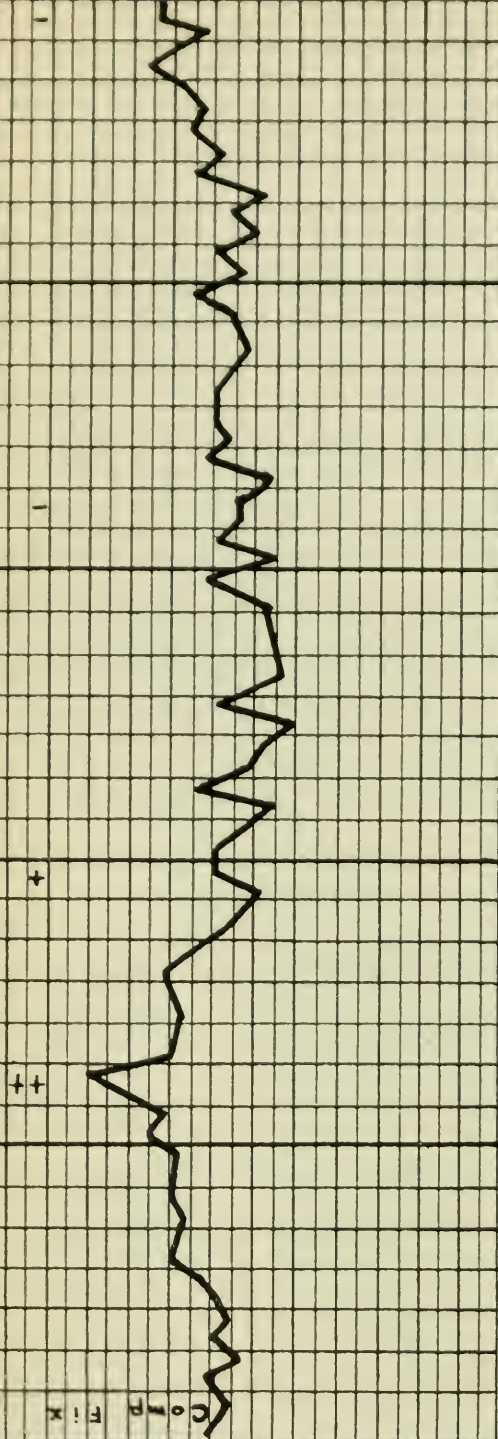
YEAR AND MONTH

DAY

TEMPERATURE FAHRENHEIT

108°  
107°  
106°  
105°  
104°  
103°  
102°  
101°  
100°  
99°  
98°  
97°

19 20 21 22 23 24 25 26 27 28 29 30 31 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22



Nov. 1915.

YEAR AND MONTH

DAY

TEMPERATURE FAHRENHEIT

106°  
105°  
104°  
103°  
102°  
101°  
100°  
99°  
98°  
97°

23 24 25 26 27 28 29 30 1 2 3 4 5 6 7 8 9 10 11 12 13

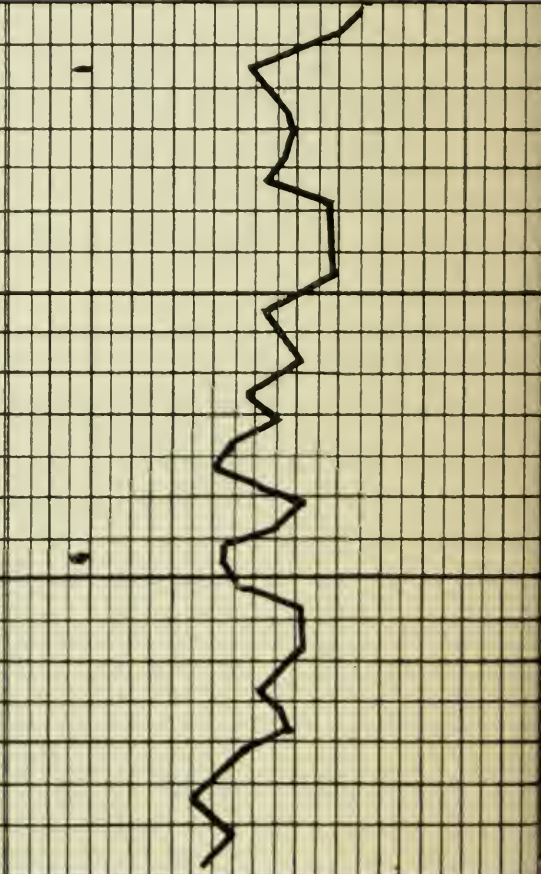


Chart 3. Clinical chart of Hog 204, showing also the results of tests for the duration of complement-binding substances in the blood of Hog 204. October 19, intramuscular injection of 5 c.c. of Virus 160 and of Virus 186. October 25, seemed well. October 27, symptoms. October 31, seemed well. November 3, normal. November 10, normal. November 20, placed in box beside radiator inside. November 24, placed in box outside and bedding kept water-soaked. December 1, cough. December 6, inactive. December 8, seemed normal. December 10, started on salt treatment. December 12, very ill. December 13, moribund.

DURATION OF COMPLEMENT-BINDING SUBSTANCES IN BLOOD OF  
IMMUNE HOGS

During the course of these experiments one naturally immune hog was found. This animal, Hog 206, was inoculated on October 28 with 2 c.c. of serum from a typical case of hog cholera in a natural field outbreak at Grosse Isle, Mich. On the 4th day after inoculation, serum from Hog 206 failed to fix complement. No symptoms of cholera appeared altho the Grosse-Isle serum was virulent for other hogs. Hog 206 was subjected to natural exposure without results, and later was used for other purposes. The serum from this animal continued to be negative in complement-fixation tests. Serum from Hog 204 was also submitted to several tests (see Chart 3).

The results of these tests on the sera of Hogs 206 and 204 indicate that complement-binding substances cease to exist in the blood of hogs when immunity against hog cholera becomes fully established.

## CONTROL ANTIGENS

In order that there might be some method of control in this work with pure *Spirochaeta-hyos* antigen, the following control antigens, prepared according to the method used in making the original spirochete antigen, were tested.

1. *B. cholera-suis* antigen from a pure culture of *B. cholera-suis* received several years ago from Theobald Smith.
2. *B. Voldagsen* antigen from a pure culture of *B. Voldagsen* received from Dr. Haendel, Königliches Hygienisches Institut, Germany, April, 1914.
3. *B. typhi-suis* (Glaesser) antigen from a pure culture of *B. typhi-suis*, also received from Dr. Haendel.
4. *Spirochaeta-hyos* Antigen 2 from a pure liquid culture from Hog 112 (New York strain).

These antigens were all prepared at the same time and tested with results as given in Table 5.

In these comparative tests with the control antigens, the maximal amounts which would not cause anticomplementary reactions were used. The results show that antigens prepared from pure cultures of *B. cholera-suis*, *B. typhi-suis*, and *B. Voldagsen*, as compared with two lots of pure *Spirochaeta-hyos* antigen, contain no specific complement-binding properties for hog-cholera serum.

A comparison of *Spirochaeta-hyos* Antigens 1 and 2, which were 3 month and 1 month old, respectively, showed the more recently prepared material to be slightly more active.

TABLE 5  
RESULTS OF COMPLEMENT-FIXATION TESTS WITH CONTROL ANTIGENS

Date of Tests	Serum Tested	Amount of Serum c.e.	Antigen	Amounts of Antigen Used, c.e.	Results
11/24	Normal B	0.04	B. Voldagsen	0.005, 0.0075, 0.01	—
11/24	Normal B	0.06	B. Voldagsen	0.005, 0.0075, 0.01	—
11/24	Normal B	0.04	Sp. hyos 2	0.005, 0.0075, 0.01	—
11/24	Normal B	0.06	Sp. hyos 2	0.005, 0.0075, 0.01	—
11/24	Cholera 187	0.04	B. Voldagsen	0.005, 0.0075, 0.01	—
11/24	Cholera 187	0.06	B. Voldagsen	0.005, 0.0075, 0.01	—
11/24	Cholera 187	0.04	Sp. hyos 2	0.005, 0.0075, 0.01	+++
11/24	Cholera 187	0.06	Sp. hyos 2	0.005, 0.0075, 0.01	++++
11/26	Normal B	0.01, 0.02, 0.03, 0.04	B. typhi-suis	0.005, 0.01	—
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	B. typhi-suis	0.005, 0.01	—
11/26	Normal B	0.01, 0.02, 0.03, 0.04	B. cholera-suis	0.005, 0.01	—
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	B. cholera-suis	0.005, 0.01	—
11/26	Normal B	0.01, 0.02, 0.03, 0.04	B. Voldagsen	0.005, 0.01	—
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	B. Voldagsen	0.005, 0.01	—
11/26	Normal B	0.01, 0.02, 0.03, 0.04	Sp. hyos 2	0.005, 0.01	—
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	Sp. hyos 2	0.005	+++
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	Sp. hyos 2	0.01	+++
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	Sp. hyos 1	0.005	+++
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	Sp. hyos 1	0.01	+++
11/26	Normal B	0.01, 0.02, 0.03, 0.04	Sp. hyos 1	0.005	—
11/26	Normal B	0.01, 0.02, 0.03, 0.04	Sp. hyos 1	0.01	—
12/ 2	Normal 204	0.06	Sp. hyos 2	0.01	—
12/ 2	Normal 204	0.06	B. Voldagsen	0.01	—
12/ 2	Normal 204	0.06	B. typhi-suis	0.01	—
12/ 2	Normal 204	0.06	B. cholera-suis	0.01	—
12/ 2	Normal 204	0.06	Sp. hyos 1	0.01	—
12/ 2	Cholera 221	0.06	Sp. hyos 2	0.01	+++
12/ 2	Cholera 221	0.06	B. Voldagsen	0.01	—
12/ 2	Cholera 221	0.06	B. typhi-suis	0.01	—
12/ 2	Cholera 221	0.06	B. cholera-suis	0.01	—
12/ 2	Cholera 221	0.06	Sp. hyos 1	0.01	+++
12/15	Cholera 215	0.06	Sp. hyos 2	0.02	+++
12/15	Cholera 215	0.06	Sp. hyos 1	0.02	++
12/15	Cholera 215	0.06	B. Voldagsen	0.02	—
12/15	Cholera 215	0.06	B. typhi-suis	0.02	—
12/15	Cholera 223	0.06	B. cholera-suis	0.02	—
12/15	Cholera 223	0.06	Sp. hyos 2	0.02	++
12/15	Cholera 223	0.06	B. Voldagsen	0.02	—
12/15	Cholera 223	0.06	B. typhi-suis	0.02	—
12/15	Cholera 223	0.06	B. cholera-suis	0.02	—
12/ 3	Normal 204	0.04	Sp. hyos 2	0.02	—
12/ 3	Normal 204	0.04	B. typhi-suis	0.02	—
12/ 3	Cholera 227	0.04	Sp. hyos 2	0.02	+++
12/ 3	Cholera 227	0.04	B. typhi-suis	0.02	—
12/ 3	Early cholera 228	0.04	Sp. hyos 2	0.02	+
12/ 3	Early cholera 228	0.04	B. typhi-suis	0.02	—
12/ 3	Early cholera 229	0.04	Sp. hyos 2	0.02	++
12/ 3	Early cholera 229	0.04	B. typhi-suis	0.02	—
12/ 3	Early cholera 230	0.04	Sp. hyos 2	0.02	+++
12/ 3	Early cholera 230	0.04	B. typhi-suis	0.02	—

CONTROL COMPLEMENT-FIXATION TESTS WITH SERA OF HOGS SUFFERING FROM DISEASES OTHER THAN HOG CHOLERA

In considering the possible specificity of the Spirocheta-hyos antigen in complement-fixation tests with hog-cholera serum, it appeared necessary to determine the results of the application of the test to sera obtained from hogs suffering from disease processes other than that of hog cholera. The committee on diseases, of the American Veterinary Medical Association, in August, 1915, reported as follows concerning the differential diagnosis of hog cholera:

Among the diseases or disease conditions that must be differentiated from hog cholera, are parasitism, a form of infectious enteritis, that condition which the U. S. Bureau of Animal Industry calls Salmonellosis and is supposed to be due to the *Bacillus suispestifer*, the so-called swine plague, pneumonia, verminous pneumonia, brine poisoning, acute pericarditis, shoat typhoid, enteritis and poisoning from spoiled foods, soap powders and irritating stock powders, swine erysipelas (which so far as we know does not exist in this country) septicemia, malignant edema, necrotic laryngitis, anthrax, heat stroke, lightning stroke, or sudden death from any cause, and a number of acute febrile conditions, that we have met with in pigs, but so far have been unable to classify.<sup>5</sup>

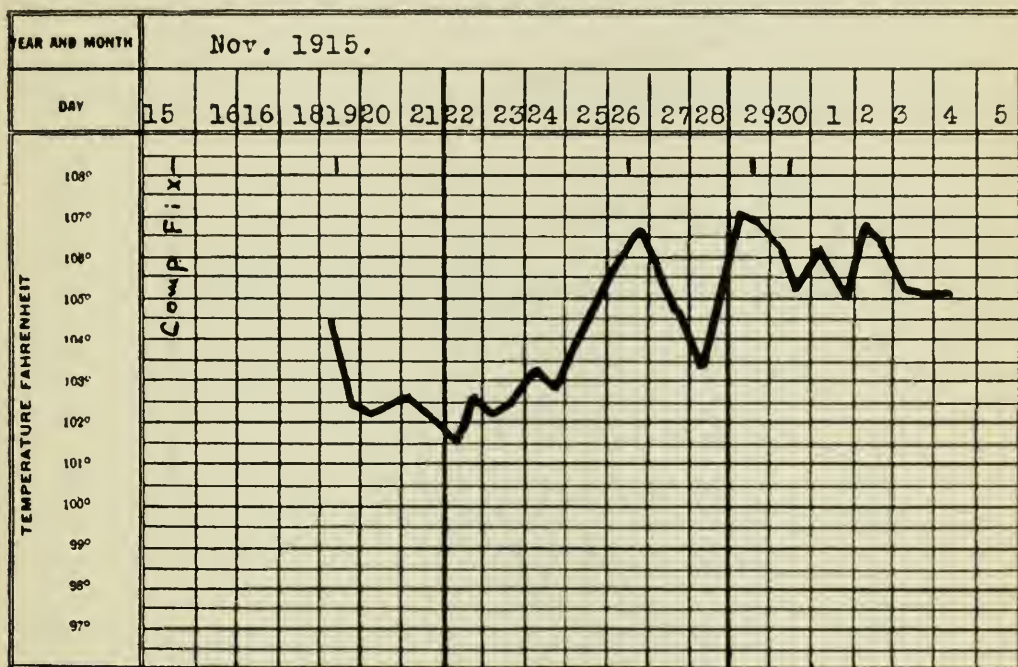


Chart 4. Clinical chart of Hog 225, which had been experimentally infected with *Staphylococcus aureus*. Results of complement-fixation tests are shown at the top of the chart. November 18, intramuscular and subcutaneous injections of 10 c.c. of a mixed broth culture of *Staph. albus*, *Staph. aureus*, and *streptococcus*. November 19, swelling at sites of injection. November 20, intramuscular injection of 10 c.c. mixed *staphylococcus* cultures Nos. 10, 15, 16 from hogs, and *pyocyaneus* (Ward). November 26, animal dull. November 29, ill. December 4, killed and examined.

In this investigation some of the foregoing pathologic conditions have been experimentally produced:

*Septicemia*.—Hog 225 (see Chart 4) exhibited typical clinical symptoms of septicemia and bacteremia. Fifteen days after the first inoculation the animal, which had been kept under carefully isolated conditions during the experiment, was killed and examined. The animal was not emaciated. There were swelling and induration at the points of inoculation. Lymphatic glands enlarged and hemorrhagic. Lungs contained a few small hemorrhagic points and one or two small areas of congestion. Heart, spleen, and liver normal. Kidneys slightly congested and covered with a few petechiae. Ental surface of bladder normal. Intestinal tract normal except for the presence of *Ascaris suum* and a slight inflammation of the mucous membrane of the large intestine.

<sup>5</sup> Jour. Am. Vet. Med. Assn., 1915, 48, p. 221.

Flask broth cultures, made from the heart blood under aseptic conditions, after 24 hours incubation yielded pure colonies of *Staphylococcus aureus* in agar transfers.

*B. Cholera-Suis Infection.*—Hog 231 (see Chart 5) showed the following: Lymphatic glands enlarged but only slightly congested. Both lungs filled with numerous small hemorrhagic areas. Heart and liver normal. Spleen normal in size, but congested in areas and soft in consistency. Kidneys congested and from 1 to 5 petechiae present. Mucosa of large intestine normal except for a few areas of ecchymosis. Ental surface of bladder normal. *B. cholera-suis* recovered in pure culture from the heart blood.

*Anthrax.*—On November 20, Hog 226 was injected intramuscularly with 2 c.c. of a 24-hour broth culture of *B. anthracis*. On November 22, as this animal showed symptoms of illness and a temperature of 104.4, a specimen of serum was collected and submitted to complement-fixation test, with negative

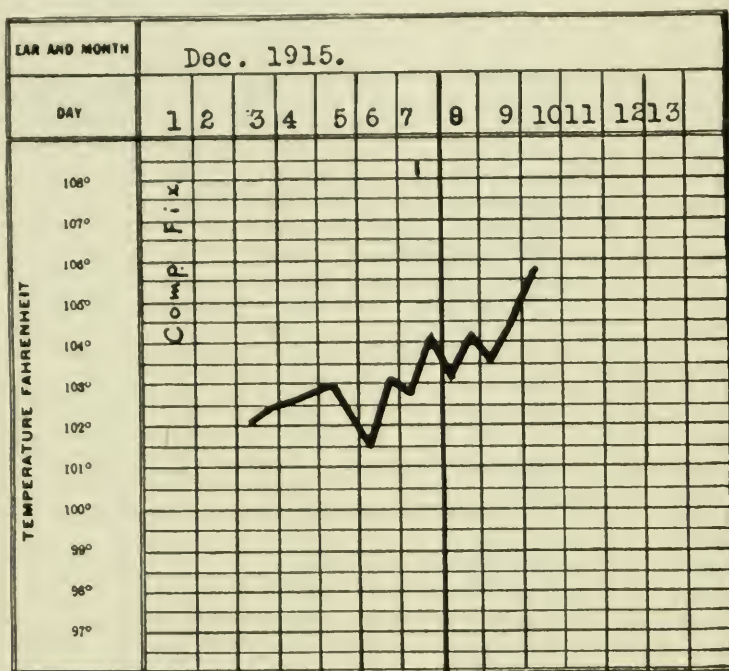


Chart 5. Clinical chart of Hog 231, which had been experimentally infected with *B. cholera-suis*. Results of complement-fixation tests shown at the top of the chart. December 1, intramuscular injection of 11 c.c. of a culture broth of *B. suispestifer* 052 (Theobald Smith). Given 5 c.c. of same cultures orally. December 7, ill. December 9, anorexia. December 10, very ill; killed and examined.

results. On November 24, the condition of Hog 226 was normal and subsequent injection with massive doses of *B. anthracis* demonstrated that the animal had acquired active immunity.

Hog 233 was injected with 20 c.c. of a virulent broth culture of *B. anthracis* on December 9. The animal, moribund on December 12, was killed; specimens of heart blood were secured for serum tests and cultures, and an autopsy made. Edematous swelling at point of inoculation. Enlarged engorged spleen. Kidneys congested. Lymphatic glands enlarged and hemorrhagic. Cultures from heart blood yielded pure *B. anthracis*. Complement-fixation tests of the serum from this animal with *Spirochaeta-hyos* antigen resulted negatively.

*Ghon-Sachs-Bacillus Infection.*—Ten cubic centimeters of a deep glucose-agar culture of the Ghon-Sachs bacillus<sup>6</sup> (original furnished by Dr. K. F. Meyer) were injected intramuscularly into Hog 232 on December 9. The following day the site of injection was surrounded by a large tender edema, the animal was inactive, and the temperature had risen to 104.6. A specimen of serum was secured from Hog 232 on December 10. The complement-fixation test resulted negatively. The 4th day after inoculation the swelling decreased, temperature fell to 102, and the animal resumed normal condition.

*Brine or Salt Poisoning and Pneumonia.*—Hog 204, immune to hog cholera (see Chart 3), was utilized for experimental brine poisoning. It will be noted in the clinical chart for this animal that an attempt was made to produce pneumonia. From November 20 to 24, Hog 204 was kept beside a warm radiator, after which it was exposed to cold and dampness. During this period the animal developed a cough, irregular temperature, and chills. Specimens of serum collected on November 22 and November 30 failed to show complement-fixation with the spirochete antigen.

From December 10 to 12, Hog 204 was given salt. On December 12 the animal showed pronounced symptoms of brine poisoning and on December 14 death occurred. A complement-fixation test with the serum secured December 12 resulted negatively. The findings at autopsy were as follows: Animal emaciated. Lungs congested, showing also large areas of gray hepatization. Lymphatic glands enlarged but not hemorrhagic. Pericarditis present. Heart enlarged. Liver mottled, engorged with blood, and enlarged. Spleen and kidneys normal in appearance. Intestinal mucosa congested.

These results show that antigen prepared from a pure culture of *Spirochaeta hyos* possesses no complement-binding properties when brought into contact with the sera of hogs suffering from septicemia (*Staph. aureus*), from infection with *B. cholera-suis*, *B. anthracis*, or the Ghon-Sachs bacillus, from brine poisoning, or from pneumonia by natural exposure.

#### DISCUSSION

In reviewing the method used in these complement-fixation tests, and in attempting to emphasize the importance of careful technic and proper controls, we wish to quote the following from a recent article by Watson:<sup>7</sup>

The successful practice of the complement-fixation test depends mainly upon the preparation and use of powerful reagents, their specificity and the accurate determination of their relative values, the fixing of standard doses wherever possible, and a constant, uniform technique and method of procedure.

Close familiarity with the activity of the reagents is essential for the best results.

Stock reagents should be prepared in quantities calculated to meet all requirements for as long a time as the activity of the reagents remains practically constant. Thus: sufficient hemolytic serum for six months' work;

<sup>6</sup> Meyer: Jour. Infect. Dis., 1915, 17, p. 458.

<sup>7</sup> Parasitology, 1915, 8, p. 156.

antigen to suffice for one month's work; fresh red cell suspension once a week; fresh complement daily or on alternate days, or as needed. It is advisable to use the blood of two sheep for sensitizing rabbits and to use the red cells of the same sheep for the hemolytic system.

The following points of extreme importance will bear repetition:

1. The amount of red cells in suspension must be very accurately measured and the standard amount never varied.
2. The use of the least possible amount of complement which with two units of hemolytic serum causes complete hemolysis of red cells.
3. The use of twice the amount of antigen which with a dourine antibody unit is necessary to fix the complement, provided the same amount of antigen alone has no inhibitory action.
4. Careful control of the inactivation of suspected sera by known positive and known negative sera.
5. Control of the diagnostic tests by a series of known positive sera, each having an antibody unit of different value, high to low.

This quotation applies equally well to the subject of complement-fixation in hog cholera except for the antibody unit of the serum, the determination of which has not been attempted.

#### CONCLUSIONS

Antigen prepared from *Spirochaeta hyos* grown in pure culture possesses well-marked specific complement-binding properties.

This antigen, when brought into contact with the sera of experimentally infected cholera hogs, produces initial complement-fixation at a period coincident with completion of the incubation period as observed in clinical conditions and thermal reactions. The specific properties of the antigen are shown to be present until death of the animal, or until active immunity is fully established.

The sera of normal hogs and those experimentally infected with *B. cholera-suis*, the Ghon-Sachs bacillus, *B. anthracis*, *Staph. aureus*, and also, the serum of one hog which was the subject of pneumonia from natural exposure, and which died from acute brine poisoning, all reacted negatively when tested for complement-fixation with *Spirochaeta hyos* antigen.

We believe that, with the observance of proper technic, the results recorded here can be duplicated without difficulty and that the method may be used to practical advantage as a reliable accurate means of laboratory diagnosis of hog cholera. Furthermore, the results of these experiments support our former conclusions that *Spirochaeta hyos* merits serious consideration as an organism possessing specific pathogenic properties in relation to hog cholera.

# OBSERVATIONS ON THE PRODUCTION OF ANTIBODIES AFTER ANTITYPHOID INOCULATION \*

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The use of antityphoid vaccine in armies and among civilians has provided opportunity for numerous observations on antibody-production. The technic used in the study of the typhoid antibodies varies so greatly that in this brief résumé of recorded observations no attempt is made to discuss the detail of methods.

## AGGLUTININ

Agglutinin has been studied more frequently than the other immune bodies that follow antityphoid inoculation. Leishman,<sup>1</sup> Wright,<sup>2</sup> Russell,<sup>3</sup> and Whitmore,<sup>4</sup> among others, made extensive series of agglutinin tests on soldiers in order to throw light on immunity against typhoid—on the question of when it appears, its duration, and other problems in connection with it. Burlingame,<sup>5</sup> and Wade and McDaniel<sup>6</sup> made similar studies in large public institutions, while Wallstein,<sup>7</sup> Moon,<sup>8</sup> Reiss,<sup>9</sup> Patrick,<sup>10</sup> Hamilton,<sup>11</sup> Harrison,<sup>12</sup> Thomsen,<sup>13</sup> Felke,<sup>14</sup> Ziersch,<sup>15</sup> Widal,<sup>16</sup> and many others studied agglutination in smaller groups.

The agglutination test has been widely employed in estimating the efficiency of vaccines. Schattstaedt<sup>17</sup> and Castellani<sup>18</sup> worked in this field.

Cummins and Cumming,<sup>19</sup> Dreyer, Walker, and Gibson,<sup>20</sup> Castellani,<sup>21</sup> and others used the agglutination test after typhoid bacilli had been injected in

\* Received for publication January 5, 1916.

<sup>1</sup> Brit. Med. Jour., 1913, 2, p. 499. Jour. Roy. Army Med. Corps, 1907, 8, p. 463; 1909, 12, p. 163.

<sup>2</sup> Studies in Immunization, 1909.

<sup>3</sup> Brit. Med. Jour., 1913, 2, p. 499. Bull. Johns Hopkins Hosp., 1910, 21, p. 83. Boston Med. and Surg. Jour., 1911, 164, p. 1.

<sup>4</sup> Bull. Johns Hopkins Hosp., 1915, 26, p. 88.

<sup>5</sup> Journal-Lancet, 1915, 35, p. 165.

<sup>6</sup> Am. Jour. Pub. Health, 1915, 5, p. 137.

<sup>7</sup> Jour. Exper. Med., 1912, 16, p. 315.

<sup>8</sup> Jour. Am. Med. Assn., 1913, 60, p. 1764. Jour. Infect. Dis., 1914, 14, p. 56.

<sup>9</sup> München. med. Wchnschr., 1915, 62, p. 1278.

<sup>10</sup> Glasgow Med. Jour., 1915, 83, p. 268.

<sup>11</sup> Tr. Chicago Path. Soc., 1912, 8, p. 151.

<sup>12</sup> Jour. Roy. Army Med. Corps, 1907, 8, p. 472.

<sup>13</sup> Hospitalstid., 1915, 58, p. 211.

<sup>14</sup> München. med. Wchnschr., 1915, 62, p. 578.

<sup>15</sup> Ibid., p. 1310.

<sup>16</sup> Presse méd., 1915, 23, p. 305.

<sup>17</sup> Jour. Am. Med. Assn., 1915, 65, p. 1713.

<sup>18</sup> Lancet, 1909, 177, p. 529.

<sup>19</sup> Jour. Roy. Army Med. Corps, 1912, 19, p. 389.

<sup>20</sup> Lancet, 1915, 188, p. 324.

<sup>21</sup> Ibid., 1913, 184, p. 595.

conjunction with other bacteria, to determine whether or not the production of specific typhoid immune bodies is modified by the presence of other bacteria. Sera from persons inoculated in this manner usually agglutinate typhoid bacilli in a dilution equal to, or higher than that employed in sera from a simple typhoid inoculation.

Results obtained in agglutination tests vary. Cahn-Bronner,<sup>22</sup> after studying 104 cases, decided that the 85th day marked a definite dividing line between the high and the low agglutinin content of the blood. Ziersch,<sup>15</sup> on the other hand, stated that the agglutinin content was subject to recognizable laws neither quantitatively nor with respect to time. However, in the literature, I found that new-formed agglutinins are recorded present in fully 90% of inoculated persons. Speaking generally, one may say that they are produced a few days after the first injection, reach their height within 1 or 2 months thereafter, and persist at least 1 year, and frequently 2 years or longer.

#### OPSONINS

Leishman<sup>23</sup> considers that the phagocytic index is the most reliable test of the value of a particular typhoid vaccine. In estimating opsonins investigators have generally employed (1) the phagocytic index and (2) the point of opsonic extinction. The latter method is considered to be more satisfactory by Russell,<sup>3</sup> Klein,<sup>24</sup> and others, because the poor staining qualities of the typhoid bacilli within the leukocytes, especially if the serum is active, make it difficult to determine an accurate phagocytic index. Klein found results practically identical whether or not the serum was heated. The period of incubation in opsonic tests has varied greatly; Klein, for example, incubating 8 minutes (heated serum), Russell (heated serum) 2 hours. The majority of investigators, among them Hamilton,<sup>11</sup> Klein,<sup>24</sup> Schattstaedt,<sup>17</sup> Russell,<sup>3</sup> and Castellani,<sup>21</sup> found the rise in opsonins to be more rapid than the rise in other antibodies, the titer higher, and the persistence shorter. Hamilton, making daily observations on opsonins, noted a negative phase, enduring from 2 to 4 days, after each injection, then a rapid rise that remained at its height about 7 days, the opsonins persisting for 2 months or longer. In the majority of cases it appears that opsonins are produced from 5 to 8 days after the first inoculation, reach their height in about 1 month, and disappear in from 4 months to 1 year.

#### BACTERIOLYSIN

Reports on the bacteriolytic power of sera from persons inoculated with typhoid vaccine for typhoid bacilli conflict. Russell,<sup>3</sup> and Cummins and Cumming<sup>19</sup> place little confidence in bacteriolytic tests because of the irregularity of the results, tho Russell concluded that a rise occurs in bacteriolysins, reaching its height 2 months after the first inoculation. Wallstein<sup>7</sup> considers these tests important. Following the method of Stern and Korte,<sup>25</sup> she found in 24 cases examined that the bacteriolytic titer was highest 2 months after the first inoculation and frequently remained above normal 1 year or more. Schattstaedt,<sup>17</sup> in a series of experiments on immunized rabbits, in which he used equal parts of progressive dilutions of serum and a 24-hour typhoid culture, plating after a 15-minute incubation, found that bacteriolysins were at their highest 6 weeks after inoculation and that they persisted for from 4 months to 1 year.

<sup>22</sup> Med. Klin., 1915, 11, p. 964.

<sup>23</sup> Lancet, 1910, 179, p. 885.

<sup>24</sup> Bull. Johns Hopkins Hosp., 1907, 18, p. 245.

<sup>25</sup> Berl. klin. Wchnschr., 1914, 41, p. 213.

Klein, by a modified Neisser method, obtained in a similar experiment the highest bacteriolytic titer 1 month after the first inoculation. After the inoculation of double (typhoid and paratyphoid) and triple (typhoid + 2 strains of paratyphoid) vaccines Widal found typhoid bacteriolysins in about the same quantities as after simple typhoid inoculation.

#### COMPLEMENT-FIXATION

Fewer observations have been made on complement-fixation than on other immune reactions after typhoid inoculations. Russell<sup>3</sup> found complement-deviation taking place in such cases, Cummins and Cumming<sup>19</sup> not. Widal, using a method similar to the Wassermann method, found inhibition of hemolysis from 8 to 13 days after the first inoculation of typhoid and paratyphoid vaccine. In some cases the reaction remained positive for 150 days. Felke<sup>14</sup> obtained complete hemolysis throughout a series of 39 cases. He concluded that complement-fixation furnishes a means of diagnosing typhoid fever in the inoculated, since the sera of patients with fever inhibited hemolysis, while sera of the inoculated did not. Hage and Korpp-Peterson<sup>26</sup> disagree with Felke; in their tests they obtained complement-fixation 8 days after inoculation. Thomsen<sup>13</sup> reports 1 strongly positive, and 8 weakly positive reactions 10 days after the second typhoid inoculation.

My own observations were made on two healthy men. Each received

#### ERRATUM

Article by Howell, Observations on the Production of Antibodies After Antityphoid Inoculation, Vol. 19, No. 1, p. 65, in the first line after the heading Agglutinin the word microscopic should have been macroscopic.

... at longer intervals. The sera were withdrawn and inactivated by heating at 56 C. for 30 minutes, and examined for agglutinin, opsonin, bacteriolysin, and complement-fixation bodies.

#### AGGLUTININ

The microscopic method was used with inactive sera and a killed suspension of a highly agglutinable typhoid strain in normal salt solution. The tubes were incubated at 37 C. for 2 hours, then kept in an ice-box overnight and readings made in the morning.

The serum from No. 1 had no typhoid agglutinins before inoculation. In 3 days after the first injection the titer was 1:40, in 19 days it had reached 1:640, and in 60 days 1:2560, the highest point. Observations were then discontinued.

<sup>26</sup> Deutsch. med. Wchnschr., 1915, 41, p. 1328.

The normal serum from No. 2 agglutinated typhoid bacilli when it was diluted 1:10. In 5 days after the first injection, the titer was 1:40, in 19 days 1:160, and in 63 days 1:5000. Agglutinins then decreased until the 134th day, when the last examination was made, and the titer found to be 1:80. In both cases there was a slight fall in agglutinins after each inoculation.

#### OPSONINS

Estimation was made by diluting the serum to the point of opsonic extinction. The dilution in which 50 leukocytes had the same percentage of cells taking part in phagocytosis as a normal control with salt solution, was considered the point of extinction. Varying dilutions of inactive sera, human leukocytes, and killed typhoid bacteria were incubated in capillary tubes at 37 C. for 15 minutes. A film was made from each tube, stained, and the number of leukocytes taking part in phagocytosis observed.

In No. 1 there was an almost immediate rise in opsonins, the titer reading 1:30 on the 3rd day, 1:20 on the 5th, and 1:240 on the 10th. There was a slightly negative phase after the second and 3rd inoculations. From the 33rd to the 60th day (marking the last observations), the titer remained constant at 1:240.

In No. 2 there was also an immediate production of opsonins. The height of the opsonic curve, 1:480, was reached on the 21st day after the 1st injection. It remained about 1:240 from the 26th to the 113th day, then fell to 1:120 on the 134th day.

#### BACTERIOLYSIN

Preliminary tests showed that 0.0125 c.c. of fresh guinea-pig serum, while having in itself little or no bactericidal effect, was sufficient to re-activate a normal inactive serum. One-tenth cubic centimeter of a 1:12 dilution of normal re-activated serum, plated after a 3-hour incubation at 37 C. with 0.02 c.c. of a typhoid suspension, gave approximately sterile plates. The typhoid suspension was of such titer that 0.1 c.c. of 0.02 c.c. typhoid suspension in 0.6 c.c. of broth plated at once gave from 500 to 600 colonies per plate.

In the bacteriolytic test, varying dilutions of each serum were placed in tubes and a fixed amount of fresh guinea-pig serum (0.0125 c.c.) and of typhoid suspension (0.02 c.c.) added to each tube. For each dilution a control tube containing a corresponding amount of inactive serum and of typhoid suspension was set up. Two other controls were also used: one of typhoid bacilli, fresh guinea-pig serum, and broth; the other of typhoid bacilli and broth only. From each of these tubes 0.1 c.c. was plated on agar at once, in order to determine whether or not the colony counts from all the tubes showed the anticipated similarity. The tubes were then incubated 3 hours at 37 C. and again 0.1 c.c. from each was plated. Counts of colonies were made after a 24-hour incubation. The highest dilution of active serum that showed a markedly lower count than the corresponding serum control, was considered to show

the amount of bacteriolysin produced. The plates made at once had from 500 to 600 colonies per plate. After a 3-hour incubation the control plates (typhoid, typhoid + complement, inactive serum + typhoid) contained 1000 or more colonies. The plates from the reactivated serum varied from sterility to 1000 or more colonies per plate.

In both Nos. 1 and 2 the titer of the sera before antityphoid inoculation was 1:48. After each inoculation a slight fall occurred in the bacteriolytic power. The highest titer reached in No. 1 was 1:384, 30 days after the first injection of vaccine. On the 60th day the titer was the same. No. 2 on the 26th day after inoculation had a titer of 1:384. After 74 days the titer decreased to 1:96 on the 113th day and 1:48 on the 134th day.

#### COMPLEMENT-FIXATION

The complement-fixation tests were made according to the technic employed in the Wassermann test (one-tenth method).

*Antigen.*—The U. S. Army vaccine was used as antigen. The anti-complementary unit was 0.2 c.c., which remained relatively stable throughout the several months in which the tests were made. Sera from convalescent typhoid patients bound complement in the presence of from 1/16 (0.01 c.c.) to 1/64 (0.005 c.c.) of this unit. Accordingly, in the tests, antigen was varied from 1/2 to 1/64 unit.

*Serum.*—The sera were inactivated by heating to 56 C. for 30 minutes. Two hundredths cubic centimeter was used in the test. Titration showed no natural antisheep amboceptor to be present in either of the sera. Fresh guinea-pig serum—0.1 c.c. of 1:10 dilution, 2 units—was used as complement.

Serum, antigen, and complement were incubated for 1 hour at 37 C.; then 0.1 c.c. of a 5% suspension of washed sheep corpuscles and 2 units of antisheep amboceptor were added to each tube and the whole incubated at 37 C. for from 15 to 30 minutes according to the controls. The customary controls (antigen, serum and hemolytic) were set up with each test, and serum from a convalescing typhoid patient was used as the positive serum control.

The serum from No. 1 showed slight binding power 14 days after the 1st injection, but was negative after the 3rd injection. It became slightly positive again on the 19th day after the 3rd injection, gradually increasing in binding power until it fixed 1/16 of the anti-complementary unit on the 33rd day. The titer was the same on the 60th day.

The serum from No. 2 began to show binding power on the 17th day after the 1st injection, reaching a maximum on the 33rd day, when it bound 1/32 of the anticomplementary unit. Binding power then decreased until there was complete absence of inhibition on the 124th day.

## SUMMARY

Agglutinin appeared in the two men on the 3rd and 5th days respectively, after the 1st inoculation, the highest point being reached in about 60 days; it was present in the serum of Patient 2 on the 134th day.

Opsonin appeared in 3 days and reached its highest concentration in 10 days in No. 1 and in 20 days in No. 2. In No. 1 opsonin was present on the 60th day and in No. 2 on the 134th day. Observations were then discontinued.

Bacteriolysin was increased on the 21st day and in both cases reached the highest point within a week.

Complement-fixation was obtained in No. 1 on the 14th day and was most marked on the 25th day. In No. 2 fixation was obtained on the 17th day and was most marked from the 29th to the 63rd days, after which the power of fixation decreased, and was wholly lost on the 124th day.

There was a slightly negative phase after each inoculation.

The severity of the general reaction in Patient 1 seemed to have no effect on antibody-production.

## CONCLUSIONS

After injection of typhoid vaccine into human beings specific antibodies develop in the blood. They reach the highest concentration in from 1 to 2 months, after which they gradually diminish. Opsonin appears to develop earliest. Agglutinin, so far as known now, persists the longest, having been demonstrated to be present 2 years and even longer after the vaccination. Specific complement-fixation is obtainable with the sera of persons injected with typhoid vaccine, hence this test is not distinctive of typhoid fever.

# THE EFFECT OF BENZENE ON THE PRODUCTION OF ANTIBODIES \*

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Selling<sup>1</sup> found that in rabbits benzene in suitable doses causes profound destruction in the hemopoietic structures, especially of the marrow, followed when recovery takes place by prompt regeneration, which is complete in from 10 to 20 days. During the height of this effect there develops a marked leukopenia, involving the granular cells relatively more than the lymphocytes. Fatty changes may develop in the kidneys and the liver, and hemorrhages in the lungs and pleura, the stomach, etc. Some reduction of red corpuscles results, embryonal red cells may appear in the blood, and the blood platelets may be reduced also,<sup>2</sup> marrow giant cells being destroyed.<sup>3</sup> In view of this more or less selective action of benzene on tissues and cells that are concerned in the production of antibodies and in the defense against infection, it lies near at hand to investigate the course of infection and the production of antibodies in animals under the influence of benzene.

Winternitz and Hirschfelder<sup>4</sup> found that rabbits in which leukopenia had been produced by means of benzene were little resistant to pneumonia from intratracheal insufflation of pneumococci, there being only very few leukocytes in the exudate. Kline and Winternitz<sup>5</sup> published further studies on the importance of the leukocytes in the reaction against pneumonia as observable in benzenized rabbits, but they did not study directly the influence of benzene on the production of pneumococcal antibodies.

White and Gammon<sup>6</sup> inoculated rabbits with tubercle bacilli and then gave benzene by way of inhalation. They found that rabbits so treated were less resistant to tuberculosis than rabbits which had not received benzene, but they offered no basis for any explanation of this effect.

In a study of the inflammatory reactions in the ears of rabbits with marked benzene leukopenia, Camp and Baumgartner<sup>7</sup> noted that there apparently was no restraint on the entrance and growth of bacteria in such animals.

\* Received for publication January 7, 1916.

<sup>1</sup> Ziegler's Beiträge, 1911, 51, p. 567.

<sup>2</sup> Duke: Arch. Int. Med., 1913, 11, p. 100. Hurwitz and Drinker: Jour. Exper. Med., 1915, 21, p. 401.

<sup>3</sup> Duke: Arch. Int. Med., 1913, 11, p. 100. E. Frank: Berl. klin. Wchnschr., 1915, 52, p. 961.

<sup>4</sup> Jour. Exper. Med., 1913, 17, p. 657.

<sup>5</sup> Ibid., 1914, 18, p. 50; 1915, 21, p. 320.

<sup>6</sup> Tr. Assn. Am. Phys., 1914, 19, p. 332.

<sup>7</sup> Jour. Exper. Med., 1915, 22, p. 174.

Schiff<sup>8</sup> found that in guinea-pigs small doses of benzene—0.01 c.c.—intra-peritoneally injected before the injection of antigen increased the reactivity to the second dose of antigen, whereas larger doses of benzene—0.03 c.c.—decreased the reactivity. Schiff mentioned several explanations but favored the view that the effect most likely is due to the action of benzene on the tissues which produce antibodies.

Rusk<sup>9</sup> was the first to publish results of direct observations on the effect of benzene on antibody-production. He recorded that in rabbits the injection of benzene in doses of 1 c.c. for each kilo of weight, either before or at the same time as the antigen, reduced greatly the formation of lysin for sheep corpuscles and of precipitin for horse serum.

Simonds and Jones<sup>10</sup> found that in rabbits injected with benzene in doses of about 1 c.c. per kilo of body weight the production of lysin for dog corpuscles and of agglutinin and opsonin for typhoid bacilli was reduced, the reduction being most marked in the case of lysin and least marked in the case of opsonin. In their work, with occasional exceptions, benzene reduced the number of leukocytes in the blood, especially the polymorphonuclears, and sooner or later evidence of damage to the erythroblastic centers appeared in the form of stippled and nucleated red cells, but in both these effects there were observed sharp individual variations.

In connection with other experiments I have been making observations for some time on the action of benzene and certain related substances on the production of antibodies.<sup>11</sup> It was thought that substances acting so directly on blood-making structures would affect the output of antibodies, in the elaboration of which these structures appear to take the leading part.

For the study of this action, benzene in an equal quantity of olive oil, was injected subcutaneously into rabbits, which had already received, or subsequently were to receive, 30 c.c. of sheep blood intra-peritoneally. Quantitative determinations were then made of the newly formed lysin and precipitin. Sheep blood in the quantity mentioned was selected for antigen because I knew from other work that usually it calls forth in otherwise normal rabbits the production of considerable, tho somewhat variable, quantities of lysin for sheep corpuscles and of precipitin for the proteins in sheep blood. Undoubtedly the injection of a considerably smaller quantity of sheep blood would have served the purpose. In order to guard against mistakes of interpretation on account of inherent variation in response to the antigen, each experiment included several animals as nearly as possible of the same age and weight, and the estimations of the antibodies studied extended over the entire period covered by the antibody curve.

<sup>8</sup> Ztschr. f. Immunitätsf., 1914, 23, p. 61.

<sup>9</sup> Univ. Calif. Publ. in Pathol., 1914, 2, p. 139.

<sup>10</sup> Jour. Med. Research, 1915, 33, p. 197.

<sup>11</sup> A brief report is published in Tr. Chicago Path. Soc., 1915, 9, p. 308.

The preparation of benzene used in all the experiments was "Benzene Merck. Highest chemical purity—crystallizable."

The details of the following experiments will serve to illustrate the methods of procedure and the principal results.

In the tables and curves the figures referring to "Lysin" represent the highest dilution of the rabbit serum in question in which distinct lysis of sheep corpuscles was produced. The tubes were incubated for 2 hours and then placed in the ice-box until the next morning. In each test the total quantity of the mixtures was 0.6 c.c., of which 0.2 c.c. was 5% suspension of sheep blood; 0.006 c.c., guinea-pig serum as complement; and the rest, rabbit serum and salt

TABLE 1

THE EFFECT ON LYSIN AND PRECIPITIN IN RABBITS, OF 8 CONSECUTIVE DAILY INJECTIONS OF 1 C.C. BENZENE, BEGINNING ON THE 4TH DAY AFTER INJECTION OF SHEEP BLOOD

Days After Injection of Sheep Blood	Rabbit 1		Rabbit 2		Rabbit 3		Control	
	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin
2	384	0	384	0	384	0	768	0
4	3072	0	3072	0	192	0	768	0
6	12288	200	6144	10	192	0	6144	50
8	12288	800	6144	400	768	50	6144	800
9	6144	200	6144	800	1536	100	6144	1600
11	6144	200	12288	200	3072	25	12288	3200
12	1536	100	768	200	384	25	12288	6400
13	1536	50	1536	100	192	25	6144	12800
15	3072	0	3072	50	Died		3072	12800
16	3072	0	3072	0			3072	12800
17	3072	.....	3072	.....	.....	.....	3072	12800
20	1536	.....	1536	.....	.....	.....	3072	12800
23	768	.....	768	.....	.....	.....	3072	12800
28	768	.....	768	.....	.....	.....	3072	12800
36	768	.....	384	.....	.....	.....	3072	3200
42	768	.....	384	.....	.....	.....	1536	1600
49	768	.....	384	.....	.....	.....	1536	800
58	48	.....	48	.....	.....	.....	96	200

The figures under "Lysin" give the highest dilution in which distinct lysis of sheep blood was produced.

The figures under "Precipitin" give the highest dilution of water-laked sheep blood with restored salt content in contact with which the rabbit serum formed a definite precipitate.

solution. Inasmuch as the estimations of lysin were made at frequent intervals during the course of an experiment, and not all at one time, the figures can not be regarded otherwise than as only approximately correct with respect to the concentration of the lysin in the rabbit serum at different bleedings because of the undoubted variations in the activity of the guinea-pig serum used as complement and in the resistance of the sheep corpuscles employed in the different sets of tests. The results are believed to be accurate enough, however, to answer the requirements in this case.

The figures in the tables and curves referring to "Precipitin" give the highest dilution of water-laked sheep blood with restored salt content in contact with which—"ring" or "zonal" method—the serum of the rabbits formed a definite precipitate after 1 hour at room temperature. As the same sheep blood was used in all the estimations, the figures may be regarded as fairly correct, especially since they practically always were made under almost identical conditions.

In the first experiment the introduction of benzene was begun 4 days after the injection of sheep blood.

Four rabbits of the same litter, weighing from 1,500 to 1,600 gm., were given each 30 c.c. of sheep blood intraperitoneally. Four days later the injection of benzene was commenced in 3 of the animals, the 4th serving as control.

TABLE 2

THE EFFECT ON LEUKOCYTES, LYSIN, AND PRECIPITIN IN RABBITS, OF 10 TO 14 CONSECUTIVE DAILY INJECTIONS OF 1 C.C. OF BENZENE, THE FIRST ON THE SAME DAY THAT 30 C.C. OF SHEEP BLOOD WERE INJECTED

Days After Injection of Sheep Blood	Rabbit 1			Rabbit 2			Rabbit 3		
	Leuko- cytes	Lysin	Precip- itin	Leuko- cytes	Lysin	Precip- itin	Leuko- cytes	Lysin	Precip- itin
1	....	....	...	....	....	...	....	....	...
2	4500	....	...	5000	....	...	3000	....	...
3	5000	....	...	4500	....	...	2800	....	...
4	3200	1536	10	4000	1536	10	2400	768	10
5	....	6144	50	....	12288	50	....	1536	50
6	3200	12288	300	3800	6144	100	2500	1536	50
7	....	6144	300	....	....	300	....	1536	300
8	3300	6144	300	3500	12288	1200	2200	1536	300
9	....	6144	300	....	6144	1200	....	....	300
10	2800	....	...	4000	12288	....	2200	1536	300
11	....	6144	600	....	6144	1200	....	3072	100
12	1800	6144	300	....	6144	1200	2600	1536	100
13	....	6144	300	....	....	1200	....	3072	100
14	....	3072	300	....	12288	1200	....	3072	100
15	2900	1536	300	3900	6144	1200	2800	3072	100
16	....	1536	300	....	....	600	....	....	...
17	....	....	...	....	....	600	....	....	...
18	2600	1536	100	5000	6144	600	3000	3072	100
19	....	....	...	....	6144	600	....	....	...
20	4800	1536	100	5400	6144	600	3200	1536	100
22	4200	1536	100	5600	6144	600	3900	1536	50
25	4000	768	100	5300	6144	300	8000	1536	50
30	4000	768	100	6000	6144	200	6400	1536	50
35	4200	768	100	5600	6144	100	4600	1536	50
40	4600	768	100	6500	3072	100	2300	3072	50
47	4425	768	100	5800	1536	100	3800	1536	50
54	....	768	100	....	3072	200	....	768	50
61	....	48	50	....	1536	1600	....	384	50
68	....	....	...	....	768	100	....	768	50
75	....	....	...	....	768	100	....	768	50
82	....	....	...	....	....	0	....	384	0
92	....	....	...	....	....	...	....	....	...

Rabbits 1, 2, and 3 received 14, and Rabbits 4 and 5, 10 injections of benzene.

The figures under "Lysin" give the highest dilution in which distinct lysis of sheep blood was produced.

Daily injections of 1 c.c. in 1 c.c. of olive oil were given subcutaneously for 8 days. The animals were bled every 2nd day or so until the 17th day, and thereafter at longer intervals, the amount of blood withdrawn each time being from 2 to 3 c.c. In the benzene rabbits the leukocytes rapidly fell to 2,000 or less, the number rising after a few days to fall again before coming to rest at about the normal level.

The results as to lysin and precipitin are given in Table 1. The chief effect of the benzene is seen to be reduction of the precipitin titer. In Rabbit 3, which died 15 days after the injection of sheep

blood, only a small amount of lysin was produced. The results obtained with the serum of the control in this experiment correspond fairly well with the titer of normal rabbits in general injected with 30 c.c. of sheep blood, the lysin titer being, however, rather lower than usual (see Control, Table 2).

TABLE 2—Continued

THE EFFECT ON LEUKOCYTES, LYSIN, AND PRECIPITIN IN RABBITS, OF 10 TO 14 CONSECUTIVE DAILY INJECTIONS OF 1 C.C. OF BENZENE, THE FIRST ON THE SAME DAY THAT 30 C.C. OF SHEEP BLOOD WERE INJECTED

Rabbit 4			Rabbit 5			Control	
Leuko- cytes	Lysin	Precip- itin	Leuko- cytes	Lysin	Precip- itin	Lysin	Precip- itin
6900	96	0	6100	96	0		
10200	24	0	13400	192	0	96	
8900	24	0	11000	192	0	768	
7000	384	0	6800	192	0	12288	
2200	6144	50	3000	1536	0	12288	400
2400	24576	50	4200	1536	25		
2400	24576	50	3100	768	50	24576	1600
3000	24576	200	4300	1536	50	49152	3200
4300	12288	800	5300	1536	50	49152	6400
3600	12288	3200	8900	1536	50		
6500	12288	3200	6100	1536	50	49152	12800
5100	12288	3200	6600	1536	0		
5100	12288	3200	6700	768	0	49152	6400
3400	6144	6400	3900	768	0		
2600	3072	3200	.....	768	0	24576	6400
.....	3072	3200	.....	768	0		
.....	.....	.....	.....	.....	0	24576	1600
.....	3072	3200	.....	768	0		
Died							
.....			.....	192	0	6144	3200
.....			.....	.....	.....	3072	800
.....			.....	.....	.....	1536	200
.....			.....	.....	.....	768	200
.....			.....	.....	.....	768	200
.....			.....	.....	.....	768	200
.....			.....	.....	.....	768	200
.....			.....	.....	.....	768	0

The figures under "Precipitin" give the highest dilution of water-laked sheep blood with restored salt content in contact with which the rabbit serum formed a definite precipitate.

In the next experiment the administration of benzene was commenced on the same day that the sheep blood was injected and then continued daily in some animals for 10 days, in others for 14 (Table 2). Practically the same results were obtained as in the first experiment; namely, marked reduction in the precipitin titer and in some rabbits reduction in lysin also. In Rabbit 4, which received 1 c.c. of benzene per kilo daily for 14 days, considerable reduction of leukocytes was observed, but in this animal the amounts of lysin and precipitin were not

much less than in many nonbenzenized rabbits. In Rabbit 5, however, which was treated in the same way, the leukocytes seem to have been reduced less and the lysin and precipitin more, a divergence of results that one might regard as due to a natural variation in the power to produce antibodies.

The following experiment illustrates the result when benzene is injected daily for some days both before and after the injection of sheep blood. The figures of Table 3 appear to show that in rabbits

TABLE 3  
THE EFFECT ON THE PRODUCTION OF ANTIBODIES OF INJECTIONS OF BENZENE (1 C.C. PER KILO)  
FOR SOME TIME BEFORE AND AFTER THE INJECTION OF THE ANTIGEN

Days After Injection of Sheep Blood	Rabbit 1			Rabbit 2			Rabbit 3			Rabbit 4		
	Leu- ko- cytes	Lysin	Pre- cip- itin	Leu- ko- cytes	Lysin	Pre- cip- itin	Leu- ko- cytes	Lysin	Pre- cip- itin	Leu- ko- cytes	Lysin	Pre- cip- itin
	5000	.....	.....	4600	.....	.....	5000	.....	.....	6100	.....	.....
	4500	.....	.....	2700	.....	.....	5000	.....	.....	4500	.....	.....
	3500	.....	.....	5300	.....	.....	6200	.....	.....	7200	.....	.....
	3700	.....	.....	2300	.....	.....	5400	.....	.....	6800	.....	.....
	4500	.....	.....	7500	.....	.....	6800	.....	.....	6600	.....	.....
	6300	.....	.....	5300	.....	.....	6300	.....	.....	5100	.....	.....
	1400	.....	.....	2000	.....	.....	6000	.....	.....	5600	.....	.....
1	3000	.....	.....	4200	.....	.....	8000	.....	.....	9600	.....	.....
2	1800	.....	.....	5400	.....	.....	4800	.....	.....	4600	.....	.....
3	5600	.....	.....	7400	.....	.....	10200	.....	.....	9400	.....	.....
4	3400	384	100	3400	192	0	8000	1536	0	6300	1536	0
5	.....	384	0	.....	192	0	.....	3072	0	.....	3072	0
6	5200	1536	50	4400	768	0	5400	6144	100	4400	6144	0
7	4000	.....	.....	4500	.....	.....	10000	.....	.....	2600	.....	.....
8	3000	1536	50	3200	1536	0	5000	6144	600	2000	3072	0
9	.....	768	100	.....	768	0	.....	6144	1600	.....	6144	1600
10	1000	.....	.....	5000	.....	.....	4400	.....	.....	2600	.....	.....
11	3400	1536	100	6000	768	0	8400	6144	1600	2400	6144	1200
12	1600	.....	.....	7200	.....	.....	.....	.....	.....	.....	Died	.....
14	.....	1536	0	.....	384	0	.....	3072	1600	.....	.....	.....
16	9000*	768	0	3000	384	0	2600	3072	800	.....	.....	.....
18	8400*	768	0	4800	384	0	5000	1536	400	.....	.....	.....
21	1800	768	0	3000	384	0	4400	1536	400	.....	.....	.....

\* Suppuration about gangrenous area in skin.  
The figures under "Lysin" give the highest dilution in which distinct lysis of sheep blood was produced.  
The figures under "Precipitin" give the highest dilution of water-laked sheep blood with restored salt content in contact with which the rabbit serum formed a definite precipitate.

so treated the amount of antibody set free may be very small, even tho the reduction of leukocytes is not extreme. The same general result is illustrated also by the record of 1 member of a group of 6, the other members of the group dying too early to permit extended observations, the results so far as they went, however, having the same bearing.

The rabbit weighed 1,030 gm. It was given 1 c.c. of benzene for 8 consecutive days; the leukocytes not falling, it was given 2 c.c. on each of the next 2 days, and for 3 days 3 c.c. each day. Two days later it received intraperitoneally 30 c.c. of sheep blood. The leukocytes remained at about 6,000

until about 5 days after the last injection of benzene; then their number fell to 3,500; subsequently the number remained between 4,000 and 5,000 for about 25 days and then rose to 6,000 or more. The amount of precipitin formed after the injection of sheep blood was insignificant; only on two days, the 6th and the 15th, did the titer reach 100. The amount of lysin produced was also small, the highest titer being 768 on the 20th day. On the 56th day after the first injection of sheep blood 30 c.c. of sheep blood were injected again and large amounts of lysin and precipitin were produced with more promptness than in the fresh animal.

TABLE 4

THE EFFECT OF BENZENE WHEN INJECTED AT THE HEIGHT OF ANTIBODY PRODUCTION

Days After Injection of 30 c.c. Sheep Blood	Injection of Benzene, c.c.	Rabbit 1		Rabbit 2		Rabbit 3	
		Lysin	Precip- itin	Lysin	Precip- itin	Lysin	Precip- itin
1	....	24	.....	0	.....	0	....
2	....	24	.....	0	.....	0	....
3	....	384	.....	384	.....	768	....
4	....	3072	.....	6144	.....	6144	....
5	....	12288	100	49152	50	12288	100
6	....	24576	400	24576	400	24576	400
7	....	12288	600	24576	600	24576	400
8	....	24576	600	24576	600	24576	1200
10	....	12288	2400	24576	600	24576	2400
11	....	12288	2400	12288	2400	24576	4800
12	0.3	6144	2400	24576	2400	12288	4800
13	0.3	12288	2400	24576	2400	12288	4800
15	0.5	3072	1600	3072	2400	1536	4800
17	0.5	768	1600	3072	3200	1536	6400
18	0.5	384	1600	3072	3200	768	6400
20	0.5	384	1600	3072	6400	768	3200
22	0.75	3072	2400	12288	9600	3072	3200
25	0.75	1536	1200	12288	9600	3072	3200
28	0.75	1536	1200	6144	4800	3072	3200
32	0.75	1536	1200	6144	2400	3072	9600
35	....	.....	1200	6144	9600	3072	36000
42	....	768	1200	3072	19200	Died	
50	....	768	1200	6144	12800		
59	....	768	1200	768	3200		
70	....	768	1200	768	4800		
77	....	768	600	768	2400		
84	....	768	300	768	600?		
94	....	768	300	768	640		
103	....	Died		768	200		
113	....			384	100		
129	....	.....	.....	384	800		
145	....	.....	.....	768	200		
161	....	.....	.....	768	200		
174	....	.....	.....	192	100?		
186	....	.....	.....	384	0		
203	....	.....	.....	384	0		
216	....	.....	.....	384	0		

The figures under "Lysin" give the highest dilution in which distinct lysis of sheep blood was produced.

The figures under "Precipitin" give the highest dilution of water-laked sheep blood with restored salt content in contact with which the rabbit serum formed a definite precipitate.

The effect of benzene on the production of antibodies when production was at or near the height of the curve, was tested also. Rabbits were injected intraperitoneally with 30 c.c. of sheep blood and on the 12th day or so injections of benzene were commenced. In the first

experiment of this kind all the animals died soon after the injections of 1 c.c. per kilo of benzene were started, and on this account in the next experiment (Table 4) the initial quantity injected was reduced to 0.3 c.c. per kilo and then gradually increased as shown in the table. There was no definite decrease in the leukocytes after the injections of benzene. The results indicate some temporary reduction of lysin in the blood but no definite effect on the precipitin. In all the animals the production of lysin and precipitin persisted longer and the precipitin underwent much more fluctuation than is the case under ordinary circumstances. Just before it died, Rabbit 3 of this experiment gave a marked sharp rise in precipitin content.

TABLE 5  
INJECTION OF BENZENE AT THE HEIGHT OF ANTIBODY PRODUCTION

Days After 2nd Injection of 30 c.c. Sheep Blood	Injections of Benzene	Lysin	Precipitin	Remarks
3	.....	12288	200	No reduction of leukocytes
5	.....	49152	800	
7	1.5 c.c. per kilo	49152	6400	
8	1.5 c.c. per kilo	49152	25600	
9*	1.5 c.c. per kilo	12288	19200	
10	1.5 c.c. per kilo			
11	2 c.c. per kilo	12288	12800	
12	2 c.c. per kilo	24576	19600	
13	2 c.c. per kilo	12288	25600	
14	.....	.....	25600	
15	.....	12288	25600	
17	.....	12288	25600	
19	.....	12288	25600	

In several other experiments of the same kind benzene has had a similar effect, even when given in much larger doses. Table 5 gives the results in a rabbit which had received 1.5 c.c., eventually 2 c.c., of benzene per kilo each day for several days during the height of the production of antibodies after a second injection of 30 c.c. of sheep blood. Reference may be made also to the injection of benzene without any recognizable effect in rabbits treated with toluene during the early stages of the production of antibodies for sheep blood. In 2 rabbits, which received 12 consecutive daily injections of toluene, approximately 1 c.c. per kilo, the 1st on the same day that 30 c.c. of sheep blood were injected, the injection some 21½ months later of 13 daily doses of 1 to 1.5 c.c. of benzene per kilo had no marked effect on the number of leukocytes, the course of antibodies, or the general health of the animals so far as ascertainable. The details of this experiment will be given later.

When doses smaller than 1 c.c. per kilo of benzene were given to rabbits injected with 30 c.c. of sheep blood, the reduction in antibodies was less pronounced, and 0.005, 0.01, and 0.025 c.c. per kilo, given daily for several days both before and after the injection of the antigen, appeared to have but little appreciable effect. There were no indications that the smaller doses just mentioned—0.01 c.c. and 0.005 c.c.—cause any stimulation of the production of antibodies so as to lead to a greater concentration in the serum than in the control rabbits even tho such doses may induce a moderate degree of leukocytosis. In dogs, however, relatively small doses of benzene appeared to increase the production of antibodies (Table 6). It would be of interest to test more thoroughly the effect of small doses of benzene when given as indicated and also during the height of the antibody curve.

TABLE 6

THE EFFECT OF BENZENE IN SMALL DOSES ON THE BLOOD AND ON THE FORMATION OF LYSIN IN THE DOG

Days After Injection of 1 c.c. of a 10% Suspension of Goat Blood per Kilo of Weight of Dog	Injections of 0.2 c.c. of Benzene per Kilo of Weight of Dog	Lysin (The figures give highest active dilution of dog serum)	Red Corpuscles	Leukocytes
	+	24		
	+	24		
1	+	24	7896000	30300
2	+	48	7598000	34450
3	+	768	8384000	29000
4		6144		
5		6144		
6	+	24576	7560000	20350
7	+	98304	6240000	27000
8	+	98304	7152000	30950
9		49152	6034000	35850
10		49152	6040000	31625
11		49152	6589000	29400
13		49152		
18		24576		
23		24576		
30		12288		

In making the tests the dog serum was heated to 58 C. for 30 minutes; each mixture measured 0.6 c.c., 0.2 c.c. being a 5% suspension of goat blood; 0.0125 c.c., guinea-pig serum as complement; the rest, dog serum and salt solution.

Besides this study on rabbits, I have made a few observations on the action of benzene on the dog and on the white rat.

In dogs the subcutaneous injection of 1 c.c. of benzene for each kilo of weight may cause at first leukocytosis, sometimes as high as 60,000 to 70,000, reduction of leukocytes taking place only after many injections and the development of fatty changes and hemorrhages. On account of the severe local and general effects no extended experiments

have been made. Table 6 gives the results of an experiment in which 0.2 c.c. of benzene per kilo of the body weight was injected several times; not only did leukocytosis develop, but the number of red corpuscles remained high and the production of lysin for goat corpuscles reached a very high point. Usually the highest point reached by the lysin after the injection of 1 c.c. of a 10% suspension of goat blood per kilo of the weight of the dog in otherwise normal animals is from 12,288 to 24,576.

In the white rat doses larger than 1 c.c. for each kilo of weight were required to depress the number of leukocytes in the blood. The most pronounced results were obtained after from 4 to 5 daily doses of from 0.6 to 0.8 c.c. of benzene with olive oil to make 2 c.c., injected subcutaneously in rats weighing from 130 to 160 gm. Usually the leukocytes began to fall after the 2nd or 3rd injection, and in a day or two the number might run less than 2,000; a gradual return to normal set in about 3 or 4 days after the injection of benzene was stopped. The injection of sheep blood (10% suspension) in the dose of 5 c.c. per kilo of weight at the end of such benzene treatment was followed by a much smaller production of specific lysin than followed in the case of rats injected with sheep blood but not with benzene. It was noted, however, that the lysin titer of the serum of benzenized rats might continue above the normal a little longer than in the untreated rats. The death rate was high in experiments on rats with benzene, even when much smaller doses than those mentioned were given.

In the course of this work many of the observations by Selling and others on the effect of benzene, in doses of 1 c.c. per kilo of weight of rabbit, on the leukocytes in the peripheral blood and on the general condition have been verified. In most cases a few injections given daily caused a distinct leukopenia, affecting the granular cells much more than the lymphocytes, and frequently also some diminution of the number of red corpuscles. In animals with extreme leukopenia the death rate was high, and in the rabbits that died while under the influence of benzene the bone marrow was poor in cells. After the first injections of benzene there sometimes occurred a preliminary leukocytosis of moderate degree. A rise in the number of leukocytes followed by a second fall before return to the normal level, as emphasized by Weiskotten, Schwartz and Steensland,<sup>12</sup> was observed in a number of animals.

<sup>12</sup> Jour. Med. Research, 1915, 33, p. 127.

In animals with distinct benzene leukopenia there was nearly always a marked reduction in the amount of lysin and precipitin, but the general form of the curves described by these substances in passing into and out of the blood resembled that of the curves in nonbenzenized rabbits, the lysin reaching the high point earlier than the precipitin, but the last phase of the curve—the gradual return to the normal—sometimes persisted longer in the benzenized animals (Charts 1 and 2).

Exceptionally rabbits were encountered in which many consecutive daily injections of 1 c.c. of benzene per kilo appeared to have little or no effect on the number of leukocytes in the peripheral blood. In these animals the relative number of granular leukocytes might be diminished while the relative number of lymphocytes was increased; in some of the resistant rabbits the production of antibodies did not seem affected nearly so much as in leukopenic rabbits.

At the height of antibody-production benzene may have little, if any, effect (Tables 4 and 5) either on antibody concentration in the serum or on the leukocytes. It appears as if rabbits at this time may have an increased resistance to benzene, and it is noteworthy too that in rabbits injected with benzene at this time, the antibodies persist longer and may undergo greater fluctuation in concentration than in rabbits not so treated.

Hamburger<sup>13</sup> finds that in the test tube small amounts of benzene promote phagocytosis, but in rabbits with benzene leukopenia, no such effect is demonstrable. As benzene leukopenia is established, the leukocytes in the blood may appear more or less injured (as observed by Smith<sup>14</sup> in the blood of leukemic patients treated with benzene) and their phagocytic activity becomes reduced, the cytophagic index for staphylococcus under the opsonic influence of normal rabbit serum having been found as low as 0.5 to 0.3 with leukocyte counts of about 1,800. Hence we may conclude that benzene may lower the anti-infectious powers of the body in at least 3 ways: by reduction of antibodies, by reduction of the number of leukocytes, and by reduction of the phagocytic activity of leukocytes. That the unguarded use of benzene in the treatment of leukemia may be associated with danger on account of its lowering the resistance to infection, is hardly to be doubted, especially in view of the course of events in some cases so treated.

The obvious explanation of the depression of antibody-formation under benzene is damage to the marrow and the lymphatic structures,

<sup>13</sup> *Lancet*, 1916, 1, p. 37.

<sup>14</sup> *Jour. Am. Med. Assn.*, 1915, 64, p. 1734.

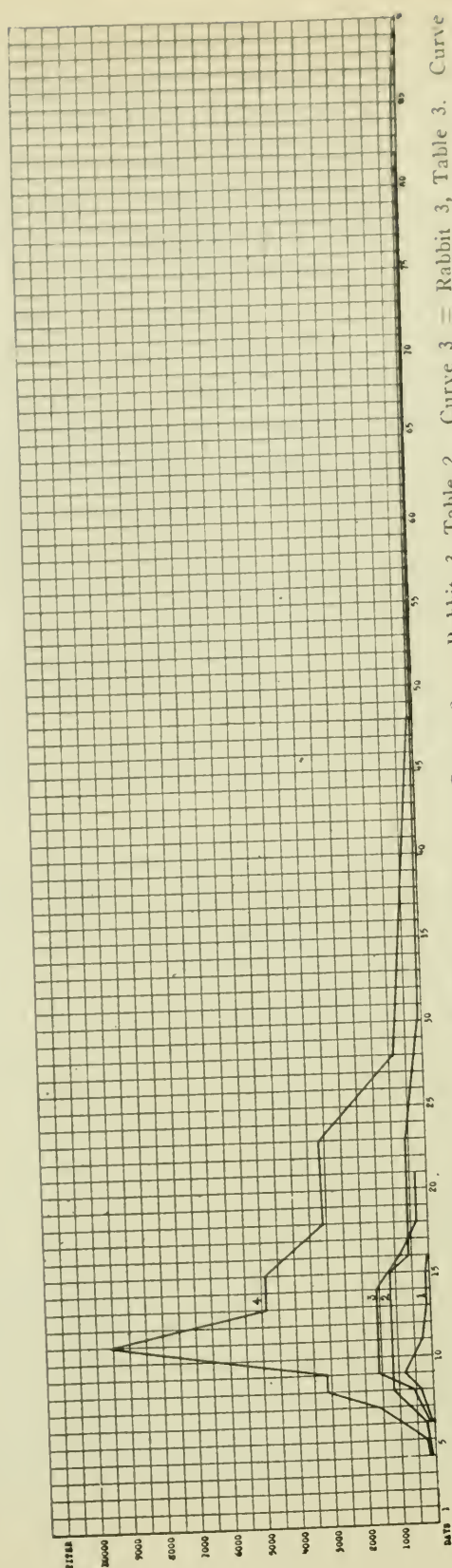


Chart 1. Precipitin Curves: Curve 1 = Rabbit 2, Table 1. Curve 2 = Rabbit 3, Table 2. Curve 3 = Rabbit 3, Table 3. Curve 4 = normal control rabbit.

which we have strong reasons to believe are concerned directly with this formation.

Pfeiffer and Marx<sup>15</sup> and others<sup>16</sup> have shown that various antibodies can be demonstrated in the bone marrow, spleen, and lymph glands before they can be demonstrated in the blood; under certain circumstances the removal of the spleen diminishes the amount of antibody produced;<sup>17</sup> when the blood-making organs are stimulated, as in acute anemia, the production of antibodies is increased; when the blood-making tissues are destroyed, as by Roentgen ray, the production is diminished;<sup>18</sup> and finally, it has been shown recently by Carrell and Ingebritson<sup>19</sup> and others<sup>20</sup> that in the presence of antigenic substances, cultures of the spleen and bone marrow outside of the body may manufacture antibodies (lysin, agglutinin, precipitin).<sup>21</sup>

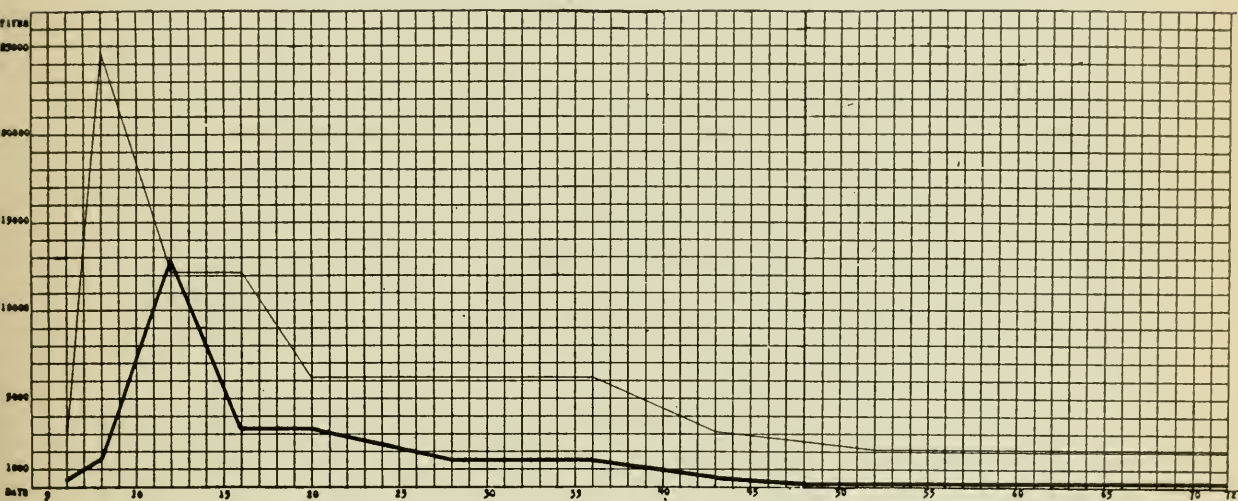


Chart 2. Lysin and Precipitin Curves: Fine line = lysin. Heavy line = precipitin.

Just how benzene interferes with the elaboration of antibodies, whether by direct injury to cells, which seems likely in view of its destructive effects on the tissues concerned, or possibly by modification of enzyme action without necessary injury to cells, are questions that at present seem difficult of solution. That benzene, in spite of its affinity for the nervous tissues according to Joachimoglu,<sup>22</sup> acts on the

<sup>15</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1898, 27, p. 272.

<sup>16</sup> Cantacuzene: Ann. de l'Inst. Pasteur, 1902, 16, p. 552; 1908, 22, p. 54. Tsurumi and Kohda: Ztschr. f. Immunitätsf., 1913, 19, p. 519.

<sup>17</sup> Deutsch: Ann. de l'Inst. Pasteur, 1899, 13, p. 689.

<sup>18</sup> Hektoen: Jour. Infect. Dis., 1915, 17, p. 415. Simonds and Jones: Jour. Med. Research, 1915, 33, p. 183.

<sup>19</sup> Jour. Exper. Med., 1912, 15, p. 287.

<sup>20</sup> Lüdke: Berl. klin. Wehnschr., 1912, 49, p. 1034. Przygode: Wien. klin. Wehnschr., 1913, 26, p. 841; 1914, 27, p. 201.

<sup>21</sup> For more complete discussion of the earlier literature see Hektoen, Harvey Society Lectures, 1909-10, p. 177, and Gay and Rusk, Tr. 15th Internat. Cong. on Demography and Hygiene, 1913, 2, p. 378.

<sup>22</sup> Biochem. Ztschr., 1915, 70, p. 93.

elements concerned in the elaboration of antibodies and that the leukocytogenic centers are concerned in this elaboration is indicated not only by the reduction of antibodies in benzene leukopenia but also, it seems to me, by the resistance to these effects of benzene at the time when the production of antibodies is at or near its height, and by the leukocytosis and increased lysin-formation observed in the dog under the influence of small doses of benzene. As pointed out by Simonds and Jones, the morphologic effects of benzene do not indicate that certain particular elements are concerned in the elaboration of antibodies to the exclusion of others, because the action is too general in that, while benzene affects the leukocytogenic centers and the leukocytes the most, it also acts, directly or indirectly, on the lymphocytes and the erythroblastic elements. The x-ray, which also reduces the production of antibodies, does not permit any morphologic differentiation because it too is diffuse in its action, altho in a different order of intensity. Possibly the study of the action of substances closely related to benzene may yield results of interest with respect to these questions. The work of Milnikowa and Wersilowa<sup>23</sup> on the action of phenylhydrazin( and of hydroxylamin hydrochlorid) on the development of agglutinins for typhoid bacilli is limited to the demonstration that the effect in the doses used is one of restraint.

The results of the investigations into antibody-formation just cited indicate that the final elaboration of antibodies takes place outside the circulation, and the results of transfusion experiments by Carlson and myself point in the same direction.<sup>24</sup> Assuming that such is the case, we have to consider the possibility that in benzenized rabbits the absorption and fixation of the antigen may be disturbed and delayed. Von Heinrich<sup>25</sup> explains the depressive action of the x-ray by assuming that it prevents the lymphatic apparatus from binding the antigen. We know that after the injection of considerable amounts, antigen may persist in the blood for some time, apparently even after the appearance of specific antibodies;<sup>26</sup> hence the question arises whether the course of the antigen in the blood differs in the case of benzenized animals from that in normal animals. I have made some observations on this point which show that in rabbits injected intraperitoneally with 30 c.c. of sheep blood the blood serum may contain sheep protein in such form as to

<sup>23</sup> *Centralbl. f. Bakteriol.*, I, O., 1912, 66, p. 520.

<sup>24</sup> *Jour. Infect. Dis.*, 1910, 7, p. 319.

<sup>25</sup> *Centralbl. f. Bakteriol.*, I, O., 1913, 70, p. 421.

<sup>26</sup> References bearing on this fact are cited by Zinsser, *Arch. Int. Med.*, 1915, 16, p. 223. See also Gay and Rusk, *Univ. Calif., Publ. in Pathol.*, 1912, 2, p. 59.

be demonstrable by means of the precipitin test for from 10 to 12 days and sometimes longer, in one instance even on the 20th day, the test in this case being made with an antiserum of a titer of 1:20000—that is, it would cause a precipitate in a dilution of sheep blood of 1:20000. I have not met any indications, however, that under the circumstances outlined, sheep protein is demonstrable by the precipitin method any longer in the blood of benzenized rabbits than in the blood of rabbits not injected with benzene, at least when antiserum of the titer of 1:12000, or thereabout, is used.

On account of its bearing on the question of what elements in the marrow and lymphatic tissues are concerned in the production of antibodies, the result of the following experiment may be of interest at this point:

In conjunction with Professor A. S. Loevenhart of the University of Wisconsin, I determined the production of lysin for sheep corpuscles in white rats which were kept in an atmosphere of 10% oxygen by means of the special apparatus devised by Professor Loevenhart to maintain atmospheres with controllable oxygen content. The rats under low oxygen pressure during the entire period of antibody-production developed an increase of hemoglobin and red cells of from 20 to 35%—total hemoglobin being increased 43%—as compared with that of the controls, but no demonstrable increase or decrease in the amount of lysin, as compared with that of controls. Hence, increase in red corpuscles under these conditions does not seem to be associated with changes in the production of antibodies as might be expected in view of the stimulating effect that acute anemia is known to have on this production; but of course without corroborative evidence this result alone, altho suggestive, does not permit one to exclude the erythrocytogenic centers from consideration as possible producers of antibodies.

The ultimate mechanism of the action of benzene, not only in restraining the production of antibodies, but in causing injury of a selective nature to marrow and lymphatic cells and leukocytes, invites further study.

#### SUMMARY

In rabbits the repeated injection of benzene in doses of 1 c.c. per kilo of rabbit weight at about the same time that sheep blood is injected greatly reduces the production of specific precipitin and lysin.

In considerably larger doses benzene has a like effect on the production of lysin in white rats.

The reduction of antibody-formation under these circumstances is associated with grave lesions in the marrow, with leukopenia, and other changes characteristic of benzene intoxication, the leukocytes in the rabbit being of reduced phagocytic power.

In the dog, benzene (0.02 c.c. per kilo) may cause leukocytosis associated with increase in production of lysin for goat corpuscles.

So far as can be determined by the precipitin method, the course of the antigen in the blood appears the same in benzenized as in non-benzenized rabbits.

At the height of antibody-production the injection of benzene appears to have but little effect on the leukocytes of the blood and on its antibody content, the precipitin especially persisting longer and with more fluctuation than otherwise.

Benzene may lower the resistance to infection by reduction (1) of antibody-production, (2) of the number of leukocytes, and (3) of leukocytic activity.

That benzene acts on elements that elaborate antibodies and that the leukocytogenic centers are concerned in this elaboration is indicated (a) in the rabbit, by the reduction of antibodies and of leukocytes and by the resistance to these effects when antibody-production is at or near its highest activity as measured by the concentration of antibodies in the blood, and (b) in the dog, when suitable doses are given, by leukocytosis and increased formation of lysin.

# THE TIME RELATIONS OF THE INFILTRATING CELLS IN ACUTE ANTERIOR POLIOMYELITIS \*

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The various phases of poliomyelitis have been studied in the human subject, and experimentally in animals. Investigations reported from the laboratories of Rockefeller Institute are notable. Comparatively recent and very complete is the work done by the committee of investigation on the New York epidemic of 1907, and also that directed by the Massachusetts state board of health in 1910 and 1911.

My work has had for its special object the determination of the time at which the different types of infiltrating cells are most evident in the lesions of the spinal cord. Material was available from 2 sets of experiments on monkeys, experiments in which the time of inoculation, and of onset of symptoms (in the greater number) were recorded. Twenty-two cases of the series were in animals used by Dr. H. J. Rosenau, and an additional 17 cases in animals used for work on poliomyelitis by Dr. H. C. Low of Boston.

The animals of the 1st group were infected by subdural injection of an emulsion of the virus contained in the spinal cord of monkeys that had died of poliomyelitis. Those of the 2nd group were injected variously—with emulsion of the spinal cord of human poliomyelitis, with material from the nasal mucous membrane of patients during the disease, and with Emulsion M. A. (Flexner and Lewis). Regardless of the different sources of the virus, similar results were obtained in all cases.

All blocks of tissue were primarily fixed in Zenker's solution and stained by the eosin methylene-blue method, after embedding in paraffin.

In the set of 21 cases in which the exact time of infection, of onset of symptoms, and of death was known, the periods were as follows:

Average length of time from infection until death.....	8-9 days
Average length of incubation period.....	4-5 days
Average time of duration of symptoms (until death).....	4 days

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The variation in each of these periods was rather wide. The shortest time between infection and death was 4 days, the longest 30 days. The shortest incubation period was 2 days, the longest 17 days. The shortest duration of symptoms before death was 1 day, the longest 24 days.

The cords showed different combinations of those lesions usually found in poliomyelitis—engorgement of the capillaries, and small perivascular hemorrhages in the gray substance. This was not an early manifestation in all instances, as these findings existed in one case in which the symptoms had persisted for 24 days. Occasional pial infiltration was seen, and perivascular dilatation with and without the presence of infiltrating cells.

Cell-infiltration existed to some extent in all sections, but it varied widely in type and degree. Three kinds of cells were distinguished: small mononuclear (lymphoid) cells, polymorphonuclear leukocytes, and large cells of the endothelioid type, frequently found where phagocytosis takes place. Sometimes all three were grouped together with slight predominance of one over the other; in other instances polymorphonuclear cells appeared almost exclusively; again, few, if any, polymorphonuclear cells were discovered among the many small mononuclear cells. It is almost always true that some polymorphonuclear leukocytes occur in sections containing the large endothelioid type of cell.

In regard to the relation of the character of the infiltrating cells to the incubation period, and the duration of symptoms, it seems to be generally true that the polymorphonuclear cells are an early manifestation, tho they may endure for some time, as was shown clearly by the fact that in those cases which developed symptoms 2 or 3 days after inoculation, many polymorphonuclear cells were found in connection with extreme degeneration of the anterior-horn cells. Their relation to these cells was not only pericellular, but also very apparently intracellular, a condition that has been previously noted by others.

The case with the shortest incubation period (2 days) in the series, in which death followed 3 days later, exhibited the infiltrating cells as a combination of small round cells and polymorphonuclear cells, with a few cells of the endothelioid type, together with extreme degeneration of the ganglion cells of the anterior horns. In direct contrast, both as to time and type of cell, was a case in which the animal lived 24 days after the onset of symptoms 6 days after inoculation. In this case the infiltrating cells were mainly small mononuclears, appearing with

extreme degeneration of the anterior-horn cells. From the sections examined the presence of polymorphonuclear cells was established to be, within certain limits, uniform with the destruction of the anterior-horn cells, the brief incubation period, and short duration of the disease followed by death. In other words, the condition apparently is governed by the toxicity of the infecting agent.

This finding was further emphasized by examination of the 17 additional cases, all showing results that corresponded in general with those of the preceding group. In these later cases, however, the average duration of symptoms, followed by death, was 26.5 days, in comparison with the 8 or 9-day period, as noted in the foregoing. The variation here, too, ranged from 96 to 6 days. This group of cases was definitely divided into 2 classes: one of long, and one of short duration. The longer varied from 17 to 96 days, the shorter from 6 to 11 days, thus coinciding with the previously described set. In all the cases of long duration, the infiltrating cells were few, with varying degrees of cell-destruction, tho never so extreme as in the 1st group. Distinct history of paralysis, however, puts them undoubtedly in the series. The group of short duration, on the other hand, shows changes wholly comparable with those of the 1st group. The infiltrating cells were a combination of the small round cells and polymorphonuclear cells, with a moderate to extreme loss of nerve cells in the anterior horns, the relation between the two varying exactly with the duration of symptoms before death.

Strauss, in his otherwise complete discussion of the histopathology of acute poliomyelitis, in the report of the New York state commission, leaves unanswered the question of the real presence of polymorphonuclear leukocytes in the cord. In my study certainly many atypical cells of variously shaped nuclei were to be found in the anterior horns, in the interstitial tissue, but they were not difficult to distinguish from the definite and easily recognizable polymorphonuclear type. They also differed distinctly from any form of proliferated glia cell which I have encountered in any instance of glia-cell increase. Morphology and staining reaction left no doubt as to their identification. The only element of difference is that Strauss worked with human material. The one human case recently at the disposal of this laboratory—one of short duration and very acute clinical history—presented a picture very similar to that of the animal cases.

One very prominent point to be noted concerning the infiltrating cells found in the cord in acute anterior poliomyelitis, was the difference between the cells about the blood-vessels and those surrounding the

degenerating anterior-horn cells. The cells about the blood-vessels in the series here considered, were almost exclusively of the small mononuclear type, even in cases where polymorphonuclear cells predominated in the anterior horns.

#### SUMMARY

Thirty-eight cases were examined, all in monkeys.

The material examined was fixed in Zenker's fluid and stained with the eosin methylene-blue stain according to Mallory.

All cases showed the usual findings in acute anterior poliomyelitis.

Cases with a short incubation period and brief duration before death, showed a corresponding abundance of polymorphonuclear cells in the pericellular infiltrate, and an extreme degeneration of anterior-horn nerve cells.

Cases in which death was delayed weeks or months showed a persistence of small round-cell infiltration, but no polymorphonuclear leukocytes, even in cases with extreme nerve-cell destruction.

#### CONCLUSIONS

The duration of the disease, the incubation period, and the cell degeneration in acute anterior poliomyelitis, apparently depend on the virulence of the infecting virus.

The polymorphonuclear leukocytes disappear after a time (in the cases here considered apparently within 3 weeks), but the small mononuclear cells, when death is sufficiently delayed, persist.

## YEASTS, PROBABLY PATHOGENIC, IN THROAT CULTURES \*

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The finding of yeast-like organisms in smears from throat cultures has been reported from time to time by observers, some of whom have considered them to be the cause of pseudomembranous angina, while others have considered them to be secondary contaminations, of no pathological significance.

In 1893 Troisier and Achalme<sup>1</sup> mentioned finding yeast-like organisms in a case of membranous angina. In 1897 De Simoni<sup>2</sup> obtained such organisms directly from 5 cases of hypertrophied tonsils. In a study of 500 cultures from patients suspected of having diphtheria De Stoecklin<sup>3</sup> found yeasts in 37 cases. In 1899 Foullerton<sup>4</sup> carefully worked out the cultural and pathogenic characteristics of various yeasts, two of which were from cases of membranous angina. He proposed the name *Saccharomyces tumefaciens albus* because culturally they gave white growths and when inoculated into guinea-pigs, caused swellings at the site of inoculation.

In 1901 Bertarelli and Calamida<sup>5</sup> found yeast-like organisms in a large percentage of individuals who themselves were not ill, but had associated with others that had membranous angina. Wilson<sup>6</sup> reported 136 cases of membranous angina in which *B. diphtheriae* was absent. In most of these cases the yeasts were present in almost pure growths. In the 12 cases which he had seen personally, the tonsils were congested, swollen, and showed a rapidly developing, thin grayish-white membrane. He also found yeasts associated with *B. diphtheriae* in 97 cases. In all instances his organisms gave a white growth, which was thick and moist, and which in fluid media fell to the bottom of the tube in a few days. They all fermented maltose and glucose, but not lactose. Breed<sup>7</sup> has also reported diphtheroid tonsillar membranous disease due to yeasts.

About 3 years ago my attention was directed to the seemingly large proportion of throat cultures in routine examinations that showed yeast-like organisms. From time to time such cultures were plated out and examined in considerable detail. As there was no attempt to

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<sup>1</sup> Arch. de méd. expér. et d'anat. path., 1893, 5, p. 29.

<sup>2</sup> Centralbl. f. Bakteriöl., 1897, 22, p. 120.

<sup>3</sup> Arch. de méd. expér. et d'anat. path., 1898, 11, p. 1.

<sup>4</sup> Jour. Path. and Bacteriol., 1899, 6, p. 37.

<sup>5</sup> Centralbl. f. Bakteriöl., I, O., 1901, 30, p. 60.

<sup>6</sup> St. Paul Med. Jour., 1904, 6, p. 649.

<sup>7</sup> Arch. Int. Med., 1912, 10, p. 108.

examine every culture of this kind, I have no statistics as to the percentage showing yeasts. In all, 90 cultures were used. Some had been sent into this laboratory for the purpose of obtaining either a diagnosis of diphtheria or a release from quarantine. Many of them

TABLE 1  
PHYSICAL CHARACTERISTICS OF CULTURES

No.	Glucose Agar	Plain Agar
1	Confluent, white, moist, glistening, and heaped up. Later, yellowish-green	A thin white film.....
2	Dull-white, dry, flat, and confluent.....	Dull-white, dry, flat, and confluent
3	Confluent, white, very moist, glistening, and heaped up. Later, yellowish-green	Rather scanty, confluent, moist, white
4	Confluent, dry, dull, cheesy, whitish, later becoming a lemon yellow	Scanty, moist, glistening, white.....
5	Very white, heaped up, dry, and glistening. Later pink	Discrete, heaped-up, white, star-shaped colonies
6	Confluent, heaped up, moist, glistening, salmon colored, and later yellow	Confluent waxy growth; along streak, pale-yellow
7	Confluent heaped-up cheesy mass, drab-white in old cultures	Confluent, moist, translucent, dirty-white
8	A confluent heaped-up growth like yellow paint. White in old cultures	Same as glucose agar.....
9	Rather scanty confluent dry yellow growth	Thin transparent yellowish film.....
10	Confluent, pink, moist, and glistening. Later dirty-gray	Same as on glucose agar.....
11	Confluent, salmon-gray, moist, paint-like along streak. Later yellow and finally white	Same as on glucose agar.....
12	Confluent, like streak of white paint. Later greenish-yellow	Same as on glucose agar only drier
13	Scanty white film, later becoming yellow, and finally brown	Scanty white growth.....
14	Confluent white veil-like growth with abrupt edges; dry partly rose-tinted	Same as on glucose agar.....
15	Confluent flat grayish growth, flows over surface of medium	Dry white film.....
16	Like bright-red paint along streaks, moist and shining. Later a gray thin film	Same as glucose agar.....
17	Like a streak of bright-yellow paint. Later grows as discrete pale-yellow colonies	Same only drier.....

Under glucose agar, the chromogenesis described as a later appearance refers to that observed after 5 subcultures had been made.

The 56 yeasts recovered are divided into the 17 varieties shown in this table as follows:

were from wholesale examinations in schools where diphtheria was epidemic.

Smears were made in the ordinary way and stained with Loeffler's methylene blue. These were kept for comparison. Then the culture

TABLE 1—*Continued*  
PHYSICAL CHARACTERISTICS OF CULTURES

Broth	Gelatin Stab	Potato
White pellicle with cuff up sides of tube	Slight growth along stab, brown	Slight, dull, confluent, dry, grayish-white
White pellicle with cuff up sides of tube	White discrete colonies along stab and heavy white growth on surface	Scanty, confluent, dull-white
At first white pellicle, later this falls to bottom. Fluid becomes clear	Slight yellowish growth along stab	A moist confluent whitish growth
Pellicle at first; later media settles clear	Slight growth along stab. Near surface, white	Dry, dull, confluent, white
Media cloudy. Slight precipitate	Same as No. 4.....	Confluent, moist, shining yellow
Media clear. Deep-brown pellicle at top	Yellowish-brown, heaped up on surface, slight along stab	Moist, glistening, yellowish-pink, confluent
White precipitate at bottom. Media clear	White on surface. No growth along stab	A heavy confluent moist white growth
Media clear; slightly brown pellicle and precipitate	Heavy growth along stab and at surface; brown	A heavy confluent salmon-colored growth
Media cloudy; brown precipitate; slight pellicle	Yellow mostly at surface	Confluent, dry, yellow
Media clear; brown precipitate; slight pellicle	White mostly at surface	Confluent, moist, shining, and gray
Media cloudy; white pellicle and white precipitate	Heavy gray growth at surface and along whole stab	Confluent gray white growth
Media clear; white precipitate and pellicle	Growth white on surface, scanty along stab	White confluent moist growth
White precipitate; medium cloudy	Very scanty white growth along stab	Scanty moist white growth
Media cloudy; white precipitate and pellicle	Slight liquefaction; brownish along stab	Confluent wet creamy growth
White precipitate; medium clear	Discrete white colonies along stab	Thin white growth, dry
Media clear; brown precipitate and pellicle	Slight pink growth along stab	Brown along streak
Media clear; yellow precipitate and pellicle	Heavy yellow growth on surface and along stab	Bright-yellow, moist, glistening, and heaped up

11 under No. 1, 4 under No. 2, 4 under No. 3, 2 under No. 4, 4 under No. 5, 4 under No. 6, 3 under No. 7, 2 under No. 8, 1 under No. 9, 2 under No. 10, 2 under No. 11, 2 under No. 12, 4 under No. 13, 3 under No. 14, 2 under No. 15, 5 under No. 16, and 2 under No. 17.

was plated out and the colonies obtained were examined. If there appeared to be yeasts, the colonies were transferred to glucose agar. From this they were grown on all the usual laboratory media, including 9 kinds of sugar-broth media in fermentation tubes.

Table 1 shows the results on ordinary media and Table 2 shows the results with special media. In only 56 instances was it possible to recover an organism that appeared to be a yeast. No organism that fermented inulin was considered to be a yeast. In 3 cases an organism of the oidium group was recovered; in 2 cases, leptothrix; in 20 cases, a mold only was recovered, and in 9 instances no fungus was recovered.

TABLE 2  
REACTIONS ON SPECIAL MEDIA

Culture No.	Dextrin	Dextrose	Galactose	Lactose	Levulose	Maltose	Mannite	Raffinose	Saccharose	Glycerin	Inulin	Indol	Nitrites
1	—	+ Gas	+	+	+	—	+	+	—	—	—	+	—
2	—	+	—	—	—	—	—	—	—	+	—	+	+
3	—	+	+	+	—	—	—	—	—	—	—	—	—
4	—	+ Gas	—	—	+ Gas	—	—	—	+	+	—	—	—
5	+	+ Gas	+	—	—	—	+	+	+	—	—	—	—
6	+ Gas	+ Gas	+ Gas	+ Gas	+ Gas	+ Gas	+ Gas	—	+ Gas	+	—	—	—
7	—	+	+	—	+	+ Gas	+	—	+	+	—	+	—
8	+ Gas	+	+ Gas	+	+ Gas	+ Gas	+ Gas	+ Gas	+ Gas	+	—	+	—
9	—	+	+ Gas	—	+	+	—	—	+	+	—	—	—
10	—	+	+	—	—	—	—	—	+ Gas	—	—	—	—
11	+	—	—	—	—	—	—	—	—	—	—	—	—
12	+	+	+	—	+	+	—	—	+	+	—	—	—
13	+	+ Gas	+	+	+ Gas	+	—	—	+	—	—	+	—
14	—	+ Gas	—	—	—	—	—	—	+	+	—	—	—
15	—	+ Gas	+	+	+ Gas	—	—	—	—	—	—	+	+
16	+	+	—	—	+	—	—	—	—	—	—	+	—
17	+	+	+	—	+	+	—	+	+	+	—	—	—

+ = acid-formation in fermentation tube or production of indol and nitrites.  
— = no change.

It is interesting to note here the recovery of a mold in such a large number of cases. However, it is well known that certain molds have a yeast-like stage, particularly the penicillium group. I have found by comparing the original smears that it is impossible to detect any difference between those that gave yeasts on the plates and those that gave molds. The question whether or not these molds have any pathological significance, has been put aside for future work.

In all, 17 distinct varieties have been recovered, as shown in Table 1.

It is still a debated question as to the importance of color in a culture of a yeast. By referring to Table 1 it is seen that often the organism changes color with a change of medium. Then, again, as in No. 14, we get two colors at the same time. Lafar<sup>8</sup> mentions certain

TABLE 3  
CLINICAL FACTS CONCERNING CASES FROM WHICH CULTURES WERE OBTAINED

Case	Number in Table 1	Laboratory Diagnosis as to Diphtheria	Remarks
1	1	+	Not obtained
2	2	—	School contact
3	3	—	Contact
4	4	+	Prolonged convalescence
5	5	—	See history of Case 1
6	6	+	Prolonged convalescence
7	7	—	Contact
8	8	—	Contact
9	9	—	Not obtained
10	10	—	Not obtained
11	11	—	Not obtained
12	6	—	Contact
13	11	—	School contact
14	14	+	Patient, a carpenter, had been repairing an old church. Convalescence very long continued. Secondary abscess
15	13	—	Contact
16	14	—	Not obtained
17	15	—	Contact
18	16	—	Tonsillitis
19	17	—	School contact
20	5	—	School contact
21	7	—	Tonsillitis
22	14	—	Contact
23	16	+	Uneventful
24	13	—	Contact
25	16	—	School contact
26	6	—	Contact
27	6	—	Contact
28	16	—	Contact
29	14	—	Contact
30	1	—	Contact
31	1	—	Pharyngitis
32	1	—	Contact
33	7	+	Delayed convalescence
34	16	—	Contact
35	2	+	Delayed convalescence
36	10	+	Enlarged tonsils with very slow recovery
37	1	—	See history of Case 2
38	5	—	See history of Case 3
39	17	—	See history of Case 4
40	5	+	Enlarged tonsils with secondary abscess
41	4	—	See history of Case 5
42	2	—	Not obtained
43	1	—	School contact
44	3	+	Uneventful
45	8	—	Tonsillitis
46	3	—	Contact
47	3	—	Tonsillitis
48	1	—	Not obtained
49	1	—	Secondary abscess
50	1	—	Not obtained
51	12	—	Uneventful
52	15	+	Not obtained
53	13	—	See history of Case 6
54	2	—	Contact
55	13	—	Contact
56	1	—	See history of Case 6

<sup>8</sup> "Technical Mycology," 6, II.

yeasts with variations in color. If we ignore this point and depend on cultural characteristics alone, the question of classification is much simplified. Also, it is not yet absolutely proved that the formation of gas in sugar media is constant with the individual yeast.

Morphologically, yeasts vary a great deal in shape and size, according to the conditions under which they are kept. Practically all work for the purpose of classifying yeasts has been done by botanists, who have based their classifications almost wholly on morphology and physical characteristics. In the present paper no attempt has been made to classify the organisms recovered or to apply to them the specific names already proposed by former workers. This work, however, is being attempted and will be published at a later date.

None of the individuals from whom the cultures were obtained has been seen clinically by the writer, but, in most cases, the clinical history has been obtained from the attending physician. Table 3 gives these clinical details briefly.

The following histories referred to in Table 3 are of interest:

CASE 5.—Woman, school-teacher, aged 20 years. Illness began Nov. 14, 1914, with a sore throat. Other members of the family had had the same trouble a short time before, and a sister who teaches in the same school had sore throat at about the same time. It was prevalent also among children in this school.

The patient showed some prostration, and had a fetid breath. Headache. Two large dark-gray membranous patches almost covered each tonsil. Pulse 120, temperature 102 F. Five hundred units of antitoxin were given. All cultures negative. Returned to work Nov. 23. On Nov. 27 the woman developed scarlet fever as did several other members of her family.

CASE 37.—Woman, who had suffered from tonsillitis for from 12 to 15 years. Illness began January 13, with a sore throat. Temperature 101, pulse 96. Both tonsils swollen and inflamed, but no exudate present. Cultures negative. The case yielded readily to ordinary treatment. Patient apparently well on 15th. On the 17th she developed a post-tonsillar abscess, which was evacuated. Recovery.

CASE 38.—Girl, aged 16. Sudden prostration with severe headache. Temperature 104.6, pulse 120, respiration 26. Two days later a small white membrane formed in the throat; very firm and difficult to remove. Culture negative. There was considerable albumin in urine. Some edema. Recovery slow.

CASE 39.—Man, taken ill Dec. 6, 1914, with chills, pain in back, and headache. Temperature slightly elevated. A post-tonsillar abscess was located and drained, after which patient recovered.

CASE 41.—This patient was a man who lived in the same household as the patient in Case 37. He presented swollen and inflamed tonsils, a temperature of 101 and a pulse of 110. Two days later, left tonsil was incised and pus evacuated. Recovery immediately followed. Cultures negative.

CASE 53.—Child, 4 years old. Temperature 101, pulse 86, respiration 24. Cervical glandular enlargement. Breathing labored and very croupy. Fetid

breath. Four days later, cheesy spots on both tonsils. Breath more fetid. Next day silvery white membrane covering both tonsils. Cultures negative. Given 4000 units of antitoxin without result. Membrane by next day had filled both nostrils, throat, and larynx. Child had to be intubated. Repeated cultures and smears were all negative. Under steam and local treatment membrane gradually disappeared in 4 days. Very little toxemia during attack.

CASE 56.—A brother of the patient in Case 41, aged 8 years. Illness started in the same way, but after the membrane had extended to the nostrils, it began to disappear. All cultures negative. Six thousand units of antitoxin given. Child not confined to bed.

From the data in Table 3 we see that 23 of the cultures were from contact cases, contracted either from suspected carriers in schools or from other cases of diphtheria in the same household. No significance at present can be given to these cases. It may be found that these yeasts are present in a large proportion of all normal throats. We do know that yeasts, as demonstrated in the case of blastomycetes, are very common in old buildings. The only instance that I have seen in which the yeasts in the throat may have come from an old building concerns a carpenter who had been repairing an old church, in whom diphtheria developed followed by a slow convalescence, yeasts of Group 14 being found in the cultures.

Ten of the cases had the yeasts associated with *B. diphtheriae*; in 7 of these we find tonsillar or other abscesses, or a prolonged convalescence, so that it really seems as if the yeasts do complicate the course of diphtheria. Whether these symbiotically render *B. diphtheriae* more resistant cannot yet be decided. It appears also that a large proportion of cases with infection and inflammation of the throat show yeasts when one would naturally expect *B. diphtheriae*; indeed, the evidence is almost conclusive that a pseudomembranous disease can be excited by these organisms. It is also noteworthy that tonsillar abscesses were present in so large a number of cases in which the yeasts were either found alone or associated with *B. diphtheriae*.

The 17 varieties of yeasts have all been inoculated subcutaneously in guinea-pigs. In every case, except Nos. 3, 5, 8, and 15, a general glandular enlargement resulted. The one inoculated with No 14, died in 3 weeks with extreme emaciation. No pathologic changes other than the glandular enlargement could be found. A yeast culturally the same as the one inoculated was recovered from the glands. The animal inoculated with No. 1 developed an abscess of one of the cervical glands, the pus of which gave a pure growth of a yeast culturally similar to the one inoculated. One other animal was killed—the one

inoculated with No. 9—and a yeast recovered from one of the glands culturally the same as the one inoculated. The other animals were still alive, about 2 months later, and still showed glandular enlargement.

In another series of guinea-pigs the inside of the cheek was scarified, and one of the cultures rubbed over the area. In all these animals, except those inoculated with Nos. 3, 5, 8, 11, 15, and 16, after about 48 hours there developed a dirty-yellowish false membrane that in the case of those inoculated with Nos. 1 and 17 extended down over the mucous membrane of the cheek, and down into the throat. The others had the membrane only over the area abraded. This membrane, which stripped very easily, on cultivation gave a yeast apparently the same as that which had been inoculated.

In conclusion, it may be said that yeasts are found in the throats of a certain percentage of all individuals, and that under proper conditions they become pathogenic, setting up either an inflammation of the mucous membranes of the throat and air-passages, or else producing a more deeply seated infection, such as a tonsillar or peritonsillar abscess. There must be long continued research before one shall be able to tell how many varieties of yeasts may be pathogenic or even to classify the present known varieties as to exact species. Experimentally, most of these organisms appear to be pathogenic for guinea-pigs. From a study of the clinical histories, I believe them to be pathogenic for man. However, as before stated, more work will have to be done before this point can be proved.

#### SUMMARY

This work has demonstrated that yeasts are a factor in the production of so-called throat infections, either alone or in association with diphtheria. It seems also that when the latter condition is present convalescence is either unusually prolonged or complicated.

The cultural characteristics of the organisms recovered from throat cultures have been worked out in considerable detail, but as yet no specific classification has been attempted.

The organisms for the most part are pathogenic for guinea-pigs both when locally applied to the abraded mucous membrane and when injected subcutaneously.

# OBSERVATIONS ON PHAGOCYTOSIS AND LEUKOCYTIC ACTIVITY IN DIPHTHERIA-CARRIERS \*

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The following experiments on phagocytosis in diphtheria-carriers were made in the hope that some light might be thrown on the mechanism whereby the bacilli are removed.

Opsonic preparations were made in the usual way. Suspensions of the leukocytes of normal persons and of diphtheria patients and carriers were made so that all should contain approximately the same number of polymorphonuclears. Various strains of diphtheria bacilli, including the strain isolated from the patient whose serum was being tested, were used; all gave similar results. The opsonic index, the cytophagic index, or the activity of the patient's leukocytes as compared with a normal person's, and the opsonic-cytophagic index, or the cytophagic power of the whole blood of the patient as compared with that of the normal control, were all determined.

The blood of each of 3 cases of diphtheria was examined in this way at frequent intervals. In one, examined at the beginning of the attack, with many bacilli free in the secretions of the nose and throat and many inside leukocytes, the serum gave an opsonic index of 1.8, a cytophagic index of 4.2, and an opsono-cytophagic index of 4.3. For several weeks a few bacilli persisted in the nose and throat, while the indices fell, remaining at about 1.3, 1.8, and 1.8, respectively; then the indices went up to 3, 1.7, and 3.2 and the bacilli disappeared from the nose and throat. Two other acute cases with low indices showed many bacilli outside the leukocytes. In 2 days the bacilli disappeared at the same time as the indices went up, the latter remaining high until about the 10th day, when they returned to normal.

The blood of 10 diphtheria-carriers (Table 1) was found to have an increased phagocytic activity. In some in which the opsonic index was normal or slightly below, the activity of the leukocytes would be high, so that the total phagocytic power of the blood was well above normal.

It is evident from these results that in carriers the phagocytic power of the blood is increased, but that at the same time the nose and

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throat may contain large numbers of leukocytes not engaged in phagocytosis, diphtheria bacilli being found outside of the leukocytes. The question arose whether the virulence of the bacilli prevents phagocytosis. In these experiments the freshly isolated virulent diphtheria bacilli were readily phagocytatable.

Various methods were then tried of increasing the activity of the leukocytes in the pharyngeal and nasal secretions. Fresh normal goat serum, which contains as much opsonin for diphtheria bacilli as is contained in normal human serum, was sprayed into the nose and throat every 2 hours for from 3 to 4 days, without, however, decreas-

TABLE 1  
PHAGOCYTOSIS IN DIPHThERIA-CARRIERS

Carrier	Opsonic Index	Cytophagic Index	Opsono-cytophagic Index
1	6.4	8.0	6.0
	5.5	7.0	11.5
	1.7	2.2	2.1
2	2.0	1.5	1.5
	2.0	1.5	3.4
	1.5	1.9	3.0
3	1.2	2.1	6.7
4	3.9	0.8	4.3
	4.9	1.3	5.8
	5.0	3.3	5.8
5	2.0	3.5	6.5
6	0.5	1.7	1.7
	0.9	1.6	1.8
	1.1	3.7	2.6
7	2.0	3.5	6.5
8	1.0	1.9	1.7
9	2.0	2.3	2.8
10	3.8	6.5	7.8
	2.0	8.3	9.8
	2.8	2.3	4.7

ing the number of diphtheria bacilli. This outcome suggested that possibly many of the leukocytes in the secretions were dead and incapable of taking part in phagocytosis. Next leukocytic suspensions were sprayed into the nose and throat every 2 hours, in the hope that fresh, actively phagocytic leukocytes might remove the bacilli, but seemingly this was also without effect.

Calcium chlorid, sodium salicylate, lactic acid, and magnesium chlorid, which have been found to increase phagocytosis, were tried also. The simplest method of testing the action of these chemicals on phagocytosis is to incubate for 15 minutes equal parts of the chemical to be tried, diluted with normal salt solution, serum, washed leukocytes, and bacteria—salt solution alone, being used in the controls. Experiments can be made also according to Hamburger's method, in

which the mixtures of serum, leukocytes, and substances to be tested are kept at room temperature, then the bacteria or carbon particles or amylum, used by Hamburger, are added, and the whole incubated. My results were alike, strong solutions of the substances inhibiting, weak ones increasing, phagocytosis.

Experiments were also made to determine whether the action of the chemical substance was on the leukocytes themselves. Equal numbers of washed leukocytes were suspended in different dilutions of the chemical for from one-half to one hour, the leukocytes were then washed by centrifugation, serum and bacteria added, and the whole incubated for 15 minutes. The dilution at which chemicals affected phagocytosis varied in different experiments, as seen in Tables 2 and 3, the phagocytosis depending evidently upon the dilution of the leukocytes in the suspension. Human serum and leukocytes were generally employed in the experiments. *Staphylococcus albus*, *Streptococcus pyogenes*, diphtheria bacilli, and a spirillum were used in these tests, which were repeated many times. These tests were made in connection with a study on phagocytic activity, which is being continued.

Hamburger<sup>1</sup> showed that calcium chlorid in a 0.005% solution has a stimulating action on phagocytosis both in vitro and in vivo. He found that a certain mineral water and Ringer's solution, both containing calcium, also promoted phagocytosis. My experiments with crystallized calcium chlorid gave results which corresponded with Hamburger's.<sup>2</sup> Emmerich and Loew report the successful use of calcium chlorid in hay fever, basing its use on its physiologic action, including increased phagocytosis. They gave one teaspoonful at meals of a solution of .100 gm. of crystallized calcium chlorid in half a liter of distilled water. I used this method with diphtheria-carriers, and also sprayed into the nose and throat a 1:200 solution, without however, producing any effect on the number of bacilli.

Altho the salicylates are used so extensively in rheumatism and other acute infections, according to Hanzlik,<sup>3</sup> who reviews completely the literature on the subject, their therapeutic action is not understood. Miller<sup>4</sup> concluded that after absorption salicylic acid circulates and appears in the tissue as a salicylate; it cannot act as a germicide unless the increased carbonic acid tension in the joint, the result of inflammation, reconverts it into salicylic acid. According to Miller, statistics

<sup>1</sup> Untersuchungen über Phagocyten, 1912, p. 91.

<sup>2</sup> München. med. Wchnschr., 1915, 62, p. 43.

<sup>3</sup> Ann. Rep. Investigations of the Therapeutic Research Committee of the Council on Pharmacy and Chemistry of the Am. Med. Assn., 1914, 3, p. 131.

<sup>4</sup> Jour. Am. Med. Assn., 1914, 63, p. 1107.

show that patients receiving salicylate are free from pain much earlier than those not so treated; that treated patients, however, relapse more frequently, so that the duration of pain and of the stay in the hospital of patients on salicylates is no shorter than in the case of patients receiving other forms of treatment, and that cardiac complications are not less frequent since the use of salicylates. He observed also that in rabbits the salicylate was of no value in preventing arthritis after intravenous injections of hemolytic streptococci. On the other hand, careful observers believe the salicylates to be of value for reasons other than their analgesic effect.

TABLE 2

PHAGOCYTOSIS IN MIXTURES OF HUMAN SERUM, HUMAN LEUKOCYTES, DIPHTHERIA BACILLI, AND SODIUM SALICYLATE

Dilution of Sodium Salicylate	Average Number of Bacilli in 50 Leukocytes	Percentage of Leukocytes Taking Part in Phagocytosis
1:100	0.48	24
1:500	0.98	46
1:1000	0.56	20
Salt solution	0.34	14

TABLE 3

PHAGOCYTOSIS IN MIXTURES OF HUMAN SERUM, HUMAN LEUKOCYTES, A SPIRILLUM, AND SODIUM SALICYLATE

Dilution of Sodium Salicylate	Average Number of Spirilla in 50 Leukocytes	Percentage of Leukocytes Taking Part in Phagocytosis
1:1000	0.72	28
1:2000	0.86	36
1:4000	0.96	46
1:8000	0.66	34
Salt solution	0.38	24

Kentzler and v. Benczur,<sup>5</sup> in their work on the action of antipyretics on phagocytosis did not observe any stimulating effect of sodium salicylate on phagocytosis, but their dilutions of 1:100 and 1:1000 may have been too low for the number of leukocytes. Jacoby and Schütze,<sup>6</sup> experimenting with rabbits, found that sodium salicylate influenced the opsonic index, causing an increase in 9 of 12 rabbits examined. I found that sodium salicylate stimulated phagocytosis in dilutions of about 1:1000 (Tables 2 and 3), and that the action was

<sup>5</sup> Ztschr. f. klin. Med., 1909, 67, p. 242.

<sup>6</sup> Biochem. Ztschr., 1908, 9, p. 527.

on the leukocytes themselves (Table 4). When strong solutions (1:5) were used, phagocytosis was inhibited. It is possible that the ability of sodium salicylate to promote phagocytosis may partly explain its beneficial action in rheumatism and other acute infections. To test these results 10-grain doses of sodium salicylate were given 3 times a day to diphtheria-carriers, without, however, any apparent effect on the number of bacilli in the nose and throat.

TABLE 4

PHAGOCYTOSIS IN MIXTURES OF HUMAN SERUM, SPIRILLA, AND LEUKOCYTES, WHICH HAD BEEN SUSPENDED FOR ONE-HALF HOUR IN SODIUM-SALICYLATE OR SALT SOLUTION AND THEN WASHED TWICE WITH SALT SOLUTION

Dilution of Sodium Salicylate	Average Number of Spirilla in 50 Leukocytes	Percentage of Leukocytes Taking Part in Phagocytosis
1:1000	1.3	56
1:2000	1.2	52
1:4000	0.72	36
1:8000	0.64	36
Salt solution	0.64	38

TABLE 5

PHAGOCYTOSIS IN MIXTURES OF HUMAN SERUM, HUMAN LEUKOCYTES, STREPTOCOCCI, AND LACTIC ACID

Dilution of Lactic Acid	Average Number of Streptococci in 50 Leukocytes	Percentage of Leukocytes Taking Part in Phagocytosis
1:20	0.04	2
1:50	2.9	42
1:100	4.1	82
1:200	3.9	68
1:400	2.4	62
1:800	2.6	56
Salt solution	2.3	60

Hektoen, quoted by Arkin,<sup>7</sup> found that lactic acid suspended phagocytosis in dilutions of 1:2000 and 1:4000. On the other hand, Bechhold<sup>8</sup> observed an increased phagocytosis with diluted lactic acid. The fact that their tests were made somewhat differently may account for the discrepancy between their results. I found that strong solutions of lactic acid suspended phagocytosis but that weak solutions (1:200) stimulated it (Table 5), and that the action was on the leukocytes themselves (Table 6). However, solutions of 1:200 applied locally in diphtheria-carriers, had no effect on the number of diphtheria bacilli. Local applications to the nose and throat are of course unsatis-

<sup>7</sup> Jour. Infect. Dis., 1913, 13, p. 408.

<sup>8</sup> München. med. Wchnschr., 1908, 55, p. 1777.

factory because one cannot reach all parts. It is also possible that the dilutions used in the test-tube experiments are quite inadequate in vivo.

Eggers<sup>9</sup> concluded that magnesium chlorid has some stimulating action on the leukocytes. Delbet's and Karajanopoulo's<sup>10</sup> experiments on dogs and in vitro appear to point out what they call the "cytophyllactic power" of a 12.1:1000 solution of anhydrous magnesium chlorid. They found it useful in dressing wounds and in subcutaneous injections. Pinard<sup>11</sup> used 18 gm. of crystallized magnesium chlorid

TABLE 6

PHAGOCYTOSIS IN MIXTURES OF HUMAN SERUM, STREPTOCOCCI, AND LEUKOCYTES, WHICH HAD BEEN SUSPENDED IN LACTIC ACID (1:200) OR SALT SOLUTION FOR ONE HOUR AND THEN WASHED TWICE WITH SALT SOLUTION

Dilution of Lactic Acid	Average Number of Streptococci in 50 Leukocytes	Percentage of Leukocytes Taking Part in Phagocytosis
1:200	11.3	64
Salt solution	5.6	40

TABLE 7

PHAGOCYTOSIS IN MIXTURES OF HUMAN SERUM, HUMAN LEUKOCYTES, STREPTOCOCCI, AND MAGNESIUM CHLORID

Dilution of Magnesium Chlorid	Average Number of Streptococci in 50 Leukocytes	Percentage of Leukocytes Taking Part in Phagocytosis
1:41	2.4	26
1:82	2.7	26
1:164	3.5	42
1:328	3.8	40
1:656	2.5	30
1:1312	1.3	20
Salt solution	0.88	18

to 1000 c.c. of sterilized water for irrigating wounds and found that healing occurred far more rapidly than with any other treatment. The results of my experiments with magnesium chlorid show that it stimulates phagocytosis considerably (Table 7). Used as a spray in a dilution of 18 gm. of the crystallized salt to 1000 c.c. of normal salt solution, in a diphtheria-carrier, it was without effect in reducing the number of bacilli, altho smears showed more phagocytosis after the spraying than before.

<sup>9</sup> Jour. Infect. Dis., 1909, 6, p. 662.

<sup>10</sup> Bull. de l'Acad. de m  d., 1915, 74, p. 266.

<sup>11</sup> Ibid., p. 577.

## SUMMARY

The phagocytic power of the blood of diphtheria-carriers is increased, but at the same time the nose and throat may contain large numbers of leukocytes not engaged in phagocytosis. Efforts to increase the activity of these leukocytes by the use of normal serum, leukocytic suspensions, calcium chlorid, sodium salicylate, lactic acid, and magnesium chlorid were unsuccessful, altho dilute solutions of these substances are found to promote phagocytosis in the test tube.



# STUDIES IN THE NITROGEN METABOLISM OF BACTERIA\*

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A chemical study of the metabolism of any organism is generally understood to mean a study of the food materials, the changes which take place in these materials within the organism, the agencies which bring about these changes, and the character and composition of the excretory products. It is obvious that the food materials of micro-organisms may be as completely studied as those of higher forms. To determine the changes these undergo within the cell, however, and the exact composition of the excretory products is a problem of a much more difficult nature than the same task would be in the case of the higher plant and animal species.

At present, students of bacterial metabolism must content themselves solely with the study of the beginning and the end of the process. What takes place within the cell can only be surmised from the nature of the enzymes which have been expressed from the bacterial bodies and from the composition of the products of bacterial action. Furthermore, it must be recognized that the substances found in a bacterial culture medium after a period of growth need not all necessarily represent the end products of metabolism. There are numerous possibilities for alteration of the true metabolic products by means of their mutual action upon one another.

In the knowledge of these difficulties, therefore, we shall have to interpret the title of this paper to mean merely a study of the nitrogenous constituents of the food supply of bacteria and a chemical examination of the products of the action of bacteria upon these food substances.

There has been no attempt on the part of investigators to make a complete study of the metabolism of any micro-organism. In fact, such a study would, in most cases, include so wide a variety of food materials and metabolic end products that the large amount of work involved would hardly be in keeping with the benefit to be gained from

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it. Knowledge of the subject has been obtained, therefore, largely by indirect routes, through researches undertaken with some other object in view.

The phenomenon of putrefaction has long been a subject for chemical research, the impetus to its investigation being derived partly from its relation to processes taking place in the intestinal tract of man and animals and partly from its relation to the preservation of food-stuffs. Earlier experiments were all of a general nature, however, being carried out either on spontaneously putrefying masses or on pure proteins inoculated from such masses. The only results were the recognition of a number of compounds as characteristic putrefactive products, and the isolation of many toxic substances. Much benefit was derived from these investigations by the subjects of medicine and biological chemistry, but little was added by them to the knowledge of bacterial metabolism. More productive in this direction has been some of the work of later years, in much of which both pure proteins and pure cultures of bacteria have been used.

The search for new and better culture media, or for media adapted to the growth of certain species of micro-organisms, has been responsible for many valuable contributions, particularly to the knowledge of what sort of nitrogenous compounds can be utilized by bacteria. Likewise, attempts to differentiate species by taking advantage of the dissimilarities in their nitrogen requirements and by noting the different products resulting from the decomposition of the same nitrogenous substance by different species, have led to a more careful investigation of nitrogen sources and of the end products of nitrogen metabolism. It is to these attempts that we owe the extensive data to be obtained on the subject of indol-formation by bacteria, as well as on their reducing and fermenting powers. In recent years the subject of creatinin-formation has been studied with the same object in view.

It is not likely that it is possible for any organism to grow and reproduce without any source of nitrogen in its food supply, tho Fermi<sup>1</sup> asserted that he had cultivated a micro-organism containing no nitrogen in its body substance. In the form, however, in which this nitrogen may be offered there is extremely wide variation. As has been known since the work of Berthelot<sup>2</sup> in 1899, there are many species which thrive with no other source of nitrogen than the uncombined atmospheric nitrogen. On the other hand, there are species which will accept such compounds as the chitin<sup>3</sup> of plant and animal origin, as their only source of this element.

And not only do we notice this variation in the sources of nitrogen among a large number of species, but even with one and the same species it is now well known that compounds of very different degrees of complexity may be utilized. The same organism may grow with a native protein, a peptone, amino-acids, amides such as urea, or even with ammonium salts, as its only source of nitrogen. Even *B. tuberculosis*, an organism formerly supposed to be exacting in its cultural requirements, has recently been grown successfully on a medium containing nitrogen only in the form of ammonium compounds.<sup>4</sup>

If bacteria show great variations in their choice of food materials, so also do they show wide differences in the ways in which they alter these materials

<sup>1</sup> Schmidt and Weis: *Die Bacterien*, 1902, p. 102.

<sup>2</sup> *Chimie vegetale et agricole*, 1899, 1.

<sup>3</sup> Benecke: *Botan. Ztg.*, 1905, 63, p. 227.

<sup>4</sup> Wherry: *Jour. Infect. Dis.*, 1913, 13, p. 144. Kendall, Day, and Walker: *Ibid.*, 1914, 15, p. 417.

in the course of metabolism, and in the kinds of chemical products which they yield by means of such alteration. Two factors must always be considered in studying the chemical products of bacterial action; namely, the species of the organism and the nature of the substance being acted upon. Upon the same substrate different species may yield very different products; likewise, as would be expected from a unicellular organism, the same species may yield quite different products when grown on media of different chemical compositions. The actual chemical processes involved in the decomposition of nitrogenous compounds by bacteria are difficult to study. Equations which have been written to represent such decompositions must, for the most part, be placed in the class of speculations. Such speculations are of great value, however, and, no doubt, frequently arrive very close to the truth.

That proteolysis through the agency of bacteria capable of attacking native proteins pursues the same general course as that brought about by the digestive enzymes of the alimentary tract of animals seems to have been established beyond dispute. Emmerling and Rieser<sup>6</sup> showed that *B. fluorescens-liquefaciens* digested gelatin with the formation of proteoses and peptones. These were later broken down to lower compounds yielding in the course of a month 25% of their nitrogen in the form of ammonia. Substitution compounds of ammonia were also found in the form of methylamin, trimethylamin, betain, and cholin. That amino-acids were an intermediary product, however, was evidenced by the fact that they were able to identify arginin and leucin. Cultures of the same organism on fibrin solutions contained tyrosin, leucin, arginin, and aspartic acid. Emmerling<sup>6</sup> identified the amino-acids, tyrosin and leucin, in cultures of virulent streptococci on blood fibrin. Mono- and trimethylamins were present here also, as well as pyridin bases. According to Taylor,<sup>7</sup> *B. coli* digests pure casein mainly to proteoses and peptones, no appreciable quantities of amino-acid being formed. On the egg-meat mixture employed by Rettger<sup>8</sup> this organism produced profound changes, giving rise to the aromatic compounds indol and skatol, the amino-acids tyrosin, leucin, and tryptophan being identified as intermediary products. Proteoses and peptones were formed also.

In the decomposition of proteins the obligate anaerobes play a most important part. In fact, according to Rettger,<sup>9</sup> true putrefactive changes with the production of the foul-smelling mercaptans and hydrogen sulfid are brought about only by this class of organisms, the part played by the aerobes and facultative anaerobes being that of creating an oxygen-free environment and removing the waste products of the strict anaerobes. From his researches it appears that *B. putrificus*, *B. oedematis*, and the bacillus of symptomatic anthrax are the most powerful putrefying organisms among the commoner anaerobes. *B. tetanus* and *B. welchii* have little or no putrefactive power, the latter being primarily a fermenting organism.

The decomposition of the primary products of protein hydrolysis by bacteria has been studied but little, altho mixtures of peptones and proteoses sold as peptone have long been the favorite basic substance in bacterial culture media. By means of the change in the rotation of polarized light, Abderhalden, Pincussohn, and Walther<sup>10</sup> studied a number of the common pathogens with

<sup>6</sup> Ber. d. deutsch. chem. Gesellschaft., 1902, 35, p. 702.

<sup>6</sup> Ibid., 1897, 30, p. 1863.

<sup>7</sup> Ztschr. f. physiol. Chem., 1902, 36, p. 487.

<sup>8</sup> Am. Jour. Physiol., 1903, 8, p. 284.

<sup>9</sup> Rettger and Newall: Jour. Biol. Chem., 1912, 13, p. 341. Rettger: Ibid., 1906, 2, p. 71; 1908, 4, p. 45.

<sup>10</sup> Ztschr. f. physiol. Chem., 1910, 68, p. 471.

respect to the extent to which they break down peptones prepared from pure proteins, and compared the results with the effect on the proteins themselves. Kendall and his co-workers<sup>11-17</sup> recently studied the production of ammonia by a large number of species, using in some cases Witte's peptone and in others a peptone solution containing meat juice. Their aim was mainly to investigate the effect that carbohydrates have on the decomposition of the nitrogenous substances. They took the ammonia-production as a measure of proteolysis. Their data show interesting exceptions to the general rule that carbohydrates have a protein-sparing effect.

The investigation by Glenn<sup>18</sup> of the inhibition of indol-formation by members of the proteus group grown in a peptone-carbohydrate solution also indicates that these compounds materially lessen proteolysis. The author attributes this effect, however, to the inactivation of the tryptic enzymes of the bacteria by the acid products of sugar-fermentation. The decreased gelatin-liquefaction by this group in the presence of sugars fermentable by them he explains in the same way.

Berghaus<sup>19</sup> also published extensive data on the subject of ammonia-formation by bacteria. He furthermore drew curves representing the production of this compound after chemical inhibition of growth.

Kendall and Farmer<sup>21</sup> attempted also to measure the rate of the production of amino-acid, but were unable with the method used (formol titration) to get results of any value.

Kendall and Walker<sup>25</sup> claimed the production of minute quantities of urea from meat-juice peptone solutions, and further stated that the amounts formed day by day were about proportional to the ammonia produced.

Antonoff,<sup>20</sup> using Weyl's test, and Germán,<sup>21</sup> using Salkowski's method, claimed creatinin-production from Witte's peptone for a large number of species. Both investigators believed the tests to have differentiating value. Fitzgerald and Schmidt<sup>22</sup> repeated these tests, but could find appreciable amounts of creatinin only in cultures of *B. proteus*. They employed both Weyl's method and Jaffé's picric-acid test.

That polypeptids are produced by bacteria has not been established as far as I know. That they may be utilized, however, as a source of nitrogen is known. Sasaki<sup>23</sup> demonstrated the ability of a variety of species to split some of the simpler peptids into their constituent amino-acids.

A study of the metabolism of bacteria grown on media containing nitrogen only in the form of amino-acids has been productive of much information that is interesting and valuable. We may deal here with synthetic as well as analytic products. That proteins are synthesized from amino-acids by micro-organisms

<sup>11</sup> Kendall and Farmer: Jour. Biol. Chem., 1912, 12, pp. 13, 19, 21, 465, 469.

<sup>12</sup> Kendall, Farmer, Bagg, and Day: Ibid., p. 219.

<sup>13</sup> Kendall and Farmer: Ibid., 1912, 13, p. 64.

<sup>14</sup> Kendall, Day, and Walker: Jour. Infect. Dis., 1913, 13, p. 425.

<sup>15</sup> Kendall and Walker: Jour. Biol. Chem., 1913, 15, p. 277.

<sup>16</sup> Kendall, Day, and Walker: Jour. Med. Research, 1913, 28, p. 465.

<sup>17</sup> Kendall, Day, and Walker: Jour. Am. Chem. Soc., 1913, 35, pp. 1201, 1208, 1211, 1217, 1225, 1237.

<sup>18</sup> Centralbl. f. Bakteriöl., I, O., 1911, 58, p. 481.

<sup>19</sup> Arch. f. Hyg., 1908, 64, p. 1.

<sup>20</sup> Centralbl. f. Bakteriöl., I, O., 1906, 43, p. 209.

<sup>21</sup> Ibid., 1912, 63, p. 545.

<sup>22</sup> Proc. Soc. Exper. Biol. and Med., 1912, 10, p. 55.

<sup>23</sup> Biochem. Ztschr., 1912, 41, p. 174; 1913, 47, pp. 462, 472.

follows as a matter of course when we say that growth is supported by them. That proteins other than those of the bacterial bodies are formed seems to be true also.<sup>24</sup> Moreover, it has been shown by Fränkel<sup>25</sup> and others<sup>26</sup> that the characteristic toxins of diphtheria and tetanus are formed in media containing only amino-acids as a source of nitrogen.

The fraction of the nitrogen of the amino-acid that is used in synthesis is always very small. The greater portion is found in the form of simpler compounds. Frouin and Ledebt<sup>27</sup> grew several species on the amino-acids resulting from the hydrolysis of serum proteins and observed that in all cases a primary acidity was produced which was followed later by strong alkalinity. Rivas<sup>28</sup> found that a short digestion of peptone with trypsin made it a much better culture medium than the undigested peptone. On such a medium he obtained indol reactions in from 5 to 6 hours.

Of the amino-acids which have been used alone as a source of nitrogen for micro-organisms, asparagin has been most studied. A very large number of organisms are capable of utilizing this compound. The main manner of decomposition is deaminization with formation of aspartic acid and a subsequent production of ammonia from the latter. That nitrogenous products other than ammonia are usually formed also is probable. Nawiasky<sup>29</sup> made a rather exhaustive study of the action of *B. proteus* on asparagin when large quantities of the organisms are added to pure asparagin solutions. The most of the nitrogen was recovered in the form of ammonia. About 5% of the asparagin which disappeared was not accounted for by the ammonia recovered.

Tyrosin is broken down by *B. coli* and yields 78.7% of the theoretical amount of p-oxyphenylethylamin.<sup>30</sup> Traetla Mosca<sup>31</sup> found another organism which decomposed this acid with the formation of p-hydrocoumaric acid and ammonia.

Of the nitrogenous compounds other than amino-acids, special interest attaches to those found in more or less abundance in the urine of man and animals. That urea, uric acid, and hippuric acid are attacked by a number of species of bacteria has long been known. Kossiwicz<sup>32</sup> showed that a number of molds were capable of utilizing these substances also. Liebert<sup>33</sup> found several varieties of bacteria that decompose uric acid to ammonia, and he stated that allantoin and urea were intermediary products. Certain other organisms have been isolated<sup>34</sup> which yield only urea from uric acid, no ammonia being formed.

That a very large number of species exist capable of converting urea to ammonium carbonate is evident from the researches of Miquel.<sup>35</sup> It is probable also that many of the common laboratory forms show this property.<sup>5</sup>

<sup>24</sup> Muller: Pflüger's Arch., 1906, 112, p. 245.

<sup>25</sup> Hyg. Rundschau, 1894, 4, p. 769.

<sup>26</sup> Uschinsky: Centralbl. f. Bakteriöl., 1893, 14, p. 316. Arch. de mèd. expèr. et d'anat. path., 1893, 5, p. 293.

<sup>27</sup> Compt. rend. Soc. de biol., 1911, 70, p. 24.

<sup>28</sup> Centralbl. f. Bakteriöl., I, O., 1912, 63, p. 547.

<sup>29</sup> Arch. f. Hyg., 1908, 66, p. 209.

<sup>30</sup> Sasaki: Biochem. Ztschr., 1914, 59, p. 429.

<sup>31</sup> Gazz. chim. Ital., 1910, 40, p. 86.

<sup>32</sup> Ztschr. Gährungsphysiol., I, 60, and II, 51.

<sup>33</sup> Botan. Centralbl., 1910, 114, p. 361.

<sup>34</sup> Ulpiani: Jahrb. f. Tierchem., 1903, 33, p. 1034. Gerard: Compt. rend., 1896, 122, p. 1019; 123, p. 185.

<sup>35</sup> Lafar's Handbuch der technischen Mykologie, 1904-1906, 3, p. 71.

Creatinin is attacked slowly by bacteria<sup>36</sup> as is creatin also. Nawiasky showed that the latter was decomposed by *B. proteus* only to the extent of 8.64%. Only 3.69% of the amount attacked was accounted for by the ammonia produced. He assumed that methylguanidin was formed.

#### THE PRODUCTION BY BACTERIA OF AMINO-ACID AND AMMONIA FROM PEPTONE

That ammonia is the chief end product of the nitrogen metabolism of bacteria seems to have been well established. That the ammonia-production by an organism growing on a protein or peptone medium is always a measure of the organism's proteolytic activity cannot, from this fact, be assumed to be true. It is quite conceivable that, because of the differences in the rate of the decomposition of the primary products of proteolysis, this criterion might lead us astray. We might, for instance, have an accumulation of amino-acids in the medium and a very slight production of ammonia, or, on the other hand, a decomposition of the amino-acids as fast as formed with a consequently high concentration of ammonia. It would give a better idea, therefore, of the rate and extent of protein-decomposition if data were secured on the concentrations of both amino-acid and ammonia. The new method originated by Van Slyke<sup>37</sup> for determining amino-acid nitrogen now makes the procuring of such data possible. The analytical results of the examination of a large number of cultures with respect to their change day by day in amino-acid and ammonia content are given in the following pages by means of tables. Some are shown also in the form of curves.

The free ammonia was determined by Folin's aeration method, in which  $\text{Ca}(\text{OH})_2$  is used to set the ammonia free from its salts. After the ammonia had been completely removed, the sample was filtered off from the excess calcium hydroxid and a determination of the amino-acid was made by Van Slyke's micro method. The Kjeldahl-Gunning-Arnold method was used for total-nitrogen determinations.

The first organisms investigated were the strongly putrefactive facultative anaerobes, *B. proteus-vulgaris* and *B. pyocyaneus*. The medium used was a solution containing 2% peptone and 0.5% NaCl. Five hundred cubic centimeters of this solution were placed in each of two 1000-c.c. flasks. After sterilization in the autoclave at 15 pounds' extra pressure, they were inoculated and placed in an incubator at 37 C. By means of a sterile pipet a sample was withdrawn from each immediately after inoculation, and at intervals of 24 hours thereafter for 11 days. These samples were analysed at once for free ammonia and amino-acids. Creatinin was also determined in the samples from the

<sup>36</sup> Ackermann: Ztschr. Biol., 1913, 62, p. 208; 63, p. 78.

<sup>37</sup> Jour. Biol. Chem., 1913, 16, p. 161.

culture of *B. proteus*. Tests for this compound in the cultures of *B. pyocyaneus* were all negative. Folin's<sup>23</sup> method was employed for the determination of creatinin.

Table 1 gives the analytical data for this test. The figures represent the total amount in milligrams of the substance mentioned at the head of the column, that is present in 100 c.c. of the culture fluid on the corresponding day. The third column under each organism gives the ratio between the amounts of amino-acid nitrogen and ammonia nitrogen present on each day of the test. Chart 1 represents the same results in the form of curves.

The data show very marked differences between the two organisms in their action on peptone solutions. In the culture of *B. proteus* we notice for the first 2 days a decrease in the amino-acid already present in the medium, followed by a rise in concentration on the 3rd, 4th and 5th days. Thereafter the concentration rises and falls without any tendency to get very far from a mean value of about 50 mgm. per 100 c.c. The ammonia nitrogen also decreases for the first 2 days, but thereafter rises rapidly until the end of the experiment, reaching the concentration of nearly 70 mgm. per 100 c.c. The ratio between the two forms of nitrogen decreases rapidly throughout the test.

The culture of *B. pyocyaneus* likewise shows an initial decrease in its amino-acid nitrogen, followed by fluctuations up and down until the 6th day, after which there occurs a gradual rise in concentration until the end of the experiment on the 10th day. The free ammonia also suffers a decrease in this culture in the first 24 hours. Thereafter there is a general tendency toward a slow increase of this substance, but in two instances, on the 4th and 7th days, a decrease occurs. The ratio, amino-acid nitrogen to ammonia nitrogen, falls in this case also, but the decline is much slower than in the case of *B. proteus*, and the ratio does not, in the time of the experiment, reach nearly so low a value. These differences are made plainer by the curves. The amino-acid curve of *B. pyocyaneus* tends to rise rapidly. The free ammonia curve of *B. proteus* rises very rapidly; that of *B. pyocyaneus* is much more gradual.

It is interesting to note that both cultures show an initial decrease in both their amino-acid nitrogen and their ammonia nitrogen, indicating that, for purposes of growth and reproduction, these organisms select the simpler forms of nitrogen in preference to the more complex peptone and proteose molecules. This seems to be in accord with the

<sup>23</sup> Jour. Biol. Chem., 1914, 17, p. 469.

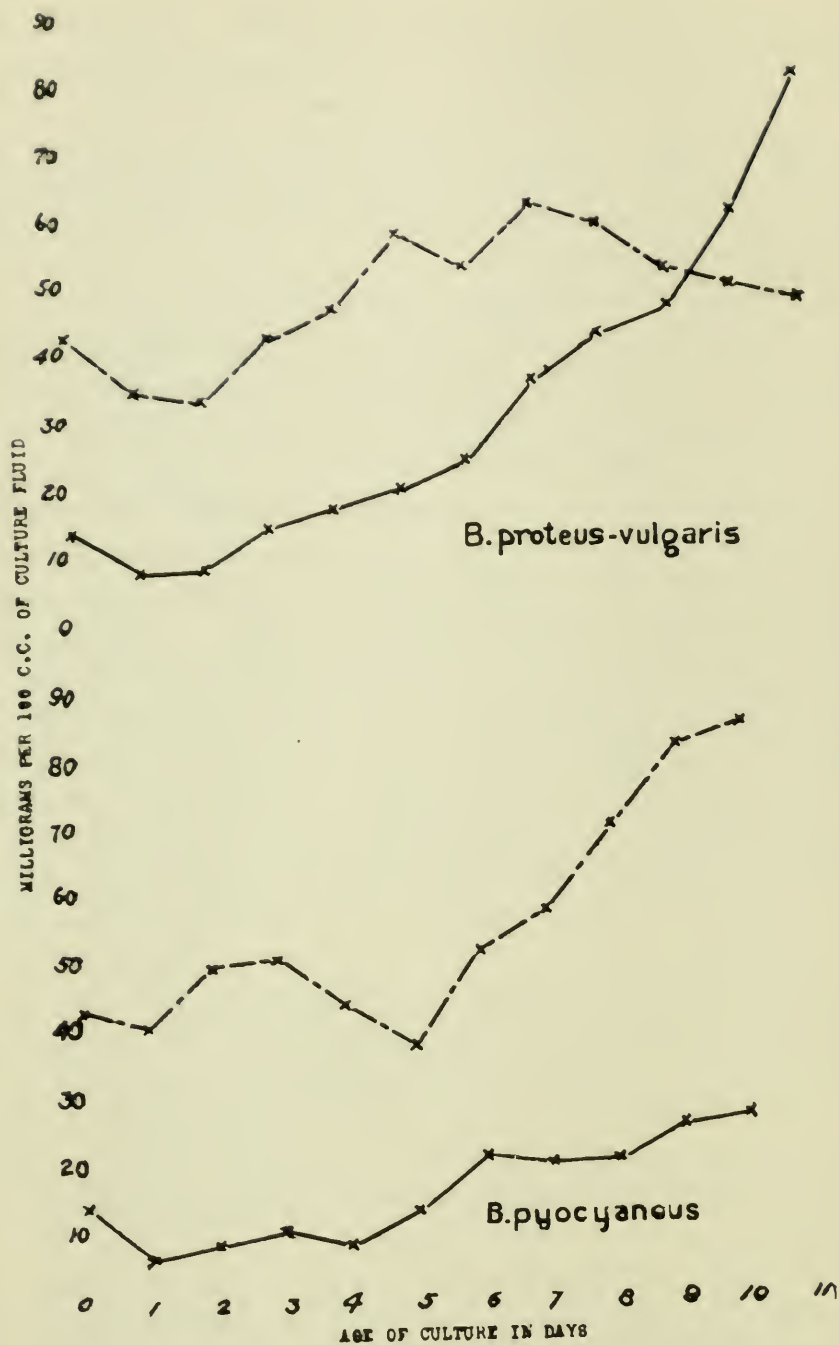


Chart 1. Production of amino-acid and ammonia by bacteria in a 2% peptone solution  
 — = ammonia nitrogen; - - - = amino-acid nitrogen.

finding of Sperry and Rettger<sup>39</sup> that pure proteins are not attacked by micro-organisms in the entire absence of simpler nitrogenous compounds.

Simultaneously with the foregoing experiment another was made using the same two organisms and also *B. coli-communis*, but employing a peptone solution containing meat juice instead of the pure peptone media. The medium contained in 1 liter the juice from 1 lb. of finely ground lean beef, 10 gm. of Witte's peptone, and 5 gm. of sodium chlorid. The reaction was made neutral to phenolphthalein. The technic of the experiment was exactly similar to that described. Table 2 and Chart 2 give the analytical results of the test. As in Table 1 the values are given in milligrams per 100 c.c. of the culture fluid.

TABLE 1

THE PRODUCTION OF AMINO-ACID AND AMMONIA BY BACTERIA IN A 2% PEPTONE SOLUTION

Age of Culture in Days	B. Proteus-Vulgaris				B. Pyocyaneus		
	Amino-acid Nitrogen	Ammonia Nitrogen	Ratio: Amino-acid N Ammonia N	Creatinin	Amino-acid Nitrogen	Ammonia Nitrogen	Ratio: Amino-acid N Ammonia N
0	42.0	13.3	4.65	—	42.0	13.3	....
1	34.0	7.3	4.52	—	39.9	5.7	7.00
2	32.9	7.3	4.52	—	48.2	7.3	6.62
3	41.8	13.0	3.22	—	49.2	9.2	5.35
4	45.3	15.6	2.91	—	42.7	7.3	5.86
5	56.5	18.2	3.10	—	36.6	12.2	3.00
6	51.2	22.7	2.26	Trace	50.5	20.0	2.53
7	60.8	34.6	1.76	Trace	56.1	19.0	2.95
8	57.9	41.4	1.40	Trace	68.8	19.5	3.54
9	51.2	45.3	1.13	Trace	80.6	24.7	3.26
10	48.9	59.6	0.82	Trace	83.7	26.0	3.22
11	46.2	69.2	0.67	Trace	....	....	....

The figures represent the amount in milligrams of the substance mentioned at the head of the column, that is present in 100 c.c. on the corresponding day.

The chief differences between the data of Table 2 and those of Table 1 are to be seen in the case of the cultures of *B. pyocyaneus*. In the presence of the muscle extractives this organism shows a considerably higher production of ammonia and a much lower production of amino-acid. There is no tendency at all toward an accumulation of the latter in the medium. Evidently in the case of this bacillus the constituents of the meat juice have a marked protein-sparing effect. The rapidly decreasing figures for creatinin indicate that this compound, at least, is easily utilized.

<sup>39</sup> Jour. Biol. Chem., 1915, 20, p. 445.

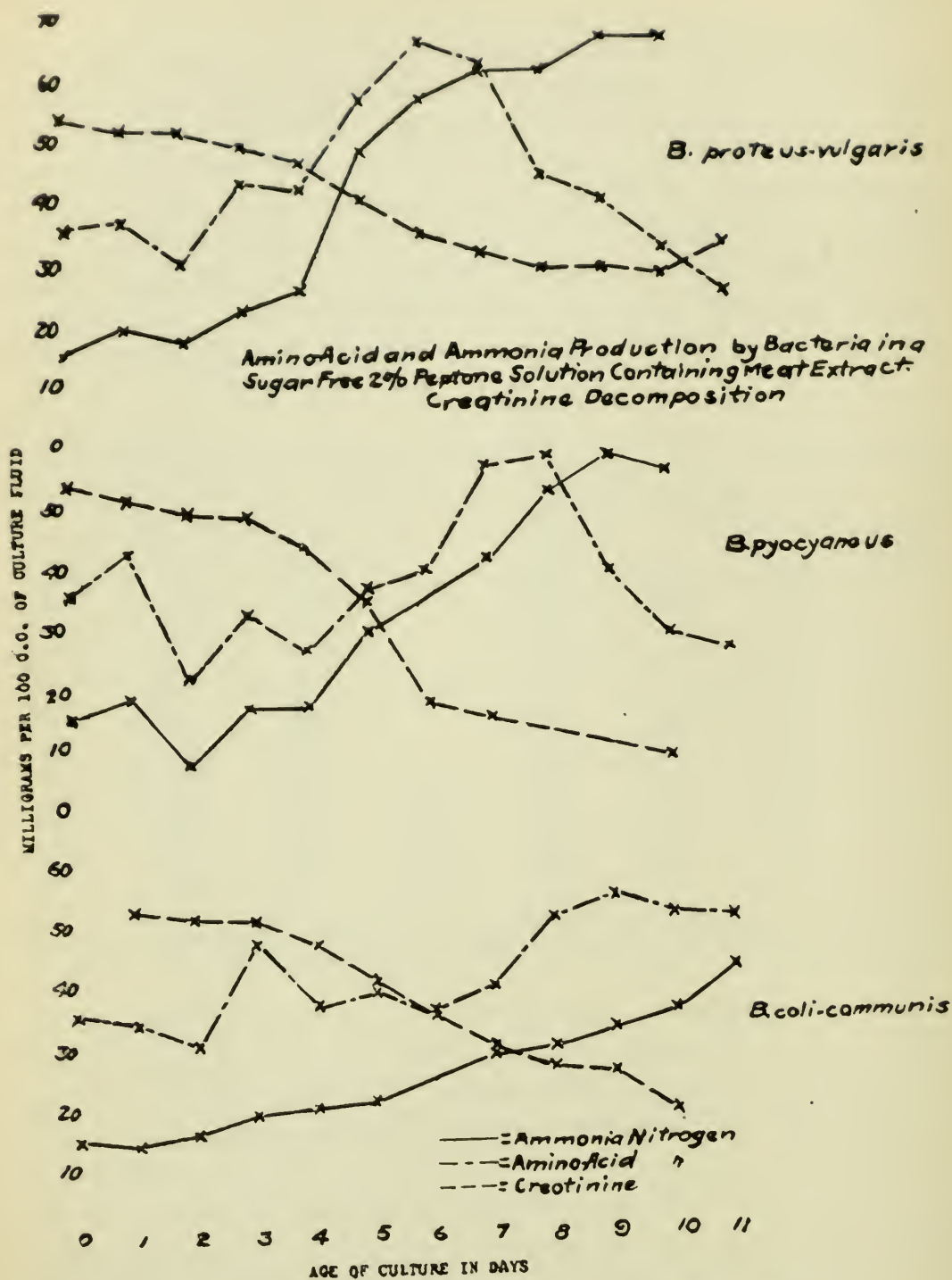


Chart 2. The key to this chart applies also to Charts 3, 4, and 5.

*B. proteus* gives very similar curves for ammonia and amino-acid, as it did in the 2% peptone solution, the ammonia values being high and the amino-acid values comparatively low, but showing, however, a tendency toward a maximum about the 6th day. On account of the ability of this organism to produce creatinin from peptone, the values for this compound cannot be taken to indicate the extent to which the extractives are utilized.

*B. coli* shows somewhat high ammonia values; in view of the comparative volumes of the cultures, they are enormously higher than the values on corresponding days shown by this organism in pure peptone solutions (see Table 4).

TABLE 2

THE PRODUCTION OF AMINO-ACID AND AMMONIA BY BACTERIA IN MEAT-EXTRACT PEPTONE SOLUTIONS

Age of Culture in Days	B. Proteus			B. Pyocyaneus			B. Coli		
	Amino-acid Nitro-gen	Am-moniam Nitro-gen	Creat-inin	Amino-acid Nitro-gen	Am-moniam Nitro-gen	Creat-inin	Amino-acid Nitro-gen	Am-moniam Nitro-gen	Creat-inin
0	35.0	14.9	53.1	35.0	14.9	53.1	35.0	14.9	53.1
1	36.1	18.7	51.2	41.8	17.9	50.7	33.6	13.5	52.7
2	29.1	16.2	51.0	21.0	6.8	48.0	29.9	15.6	51.2
3	42.4	21.1	48.5	31.4	16.2	47.8	46.7	18.4	50.6
4	41.6	24.7	45.8	25.3	16.2	42.7	36.0	19.3	46.5
5	55.9	47.4	39.4	35.2	28.4	33.1	38.5	20.6	40.1
6	65.1	56.0	33.8	38.4	33.9	16.2	35.3	24.2	35.0
7	61.7	60.2	30.3	55.1	39.8	13.6	39.5	28.4	29.8
8	42.8	60.2	27.6	56.6	50.8	11.7	50.6	29.6	26.1
9	38.5	65.7	27.6	37.6	56.5	9.2	54.1	32.5	25.2
10	30.1	65.0	26.1	27.2	53.6	7.0	51.2	35.4	18.6
11	23.1	....	31.0	24.4	52.1	6.0	50.9	42.2	16.1

As none of the organisms used in the described tests reached the limits of its chemical activity in the time of the experiment, it was thought worth while to repeat the experiment, continuing it over a longer period. Furthermore, as it was thought possible that the removal of samples with a pipet every 24 hours might introduce errors by disturbing the culture or through failure to take account of slight differences that might exist in the concentration of the substances determined in different layers of the solution, the following rather tedious method was used:

A 2% peptone solution containing 0.5% NaCl was made up and placed in 40-c.c. portions in small Erlenmeyer flasks. These, after sterilization in the autoclave, were all placed in the incubator at 37 C. Each day 2 flasks were inoculated with 1 loopful of a 24-hour peptone culture of *B. proteus* and *B. pyocyaneus*, respectively. In this way separate cultures were obtained ranging in age from 1 to 28 days, all of which had been inoculated with approximately

the same number of organisms and kept for the full time of the experiment under exactly the same conditions. When all but the control flask had been inoculated, they were sterilized by the addition of about 2% phenol. Two cubic centimeters of N/1 HCl were added to each and the cultures filtered. The filtrate, which was fairly clear, was made up to volume and aliquot parts taken for the determinations. Table 3 gives the analytical results. As in Tables 1 and 2, the values are given in milligrams per 100 c.c. of the culture medium.

TABLE 3  
THE PRODUCTION OF AMINO-ACID AND AMMONIA BY BACTERIA IN A PEPTONE SOLUTION

Age of Culture in Days	B. Proteus-Vulgaris					B. Pyocyaneus		
	Amino-acid Nitrogen	Ammonia Nitrogen	Ratio: Amino-acid N	Creatinin	Creatin	Amino-acid Nitrogen	Ammonia Nitrogen	Ratio: Amino-acid N
			Ammonia N					Ammonia N
0	37.0	6.5	5.70	.....	....	37.0	6.5	5.70
1	62.0	24.2	2.56	.....	....	44.4	14.5	3.06
2	60.0	25.0	2.40	Trace	....	89.4	21.8	4.10
3	54.5	40.5	1.34	Trace	6.3	112.0	28.1	5.14
4	60.0	40.5	1.48	Trace	10.5	63.1	25.4	2.48
5	56.5	53.7	1.05	Trace	....	84.3	28.1	3.00
6	59.7	59.0	1.01	Trace	....	80.0	31.8	2.52
7	43.2	81.2	0.54	Trace	....	116.0	37.2	3.12
8	52.5	96.2	0.55	Trace	14.0	59.8	39.8	1.50
9	39.5	92.5	0.43	6.1	....	120.0	51.0	2.36
10	39.5	81.5	0.49	6.0	....	59.2	46.7	1.27
11	32.5	104.0	0.32	6.5	....	101.0	65.7	1.54
12	37.0	103.0	0.35	6.5	16.5	53.0	43.8	1.21
13	....	120.0	....	6.5	....	70.5	47.9	1.47
14	27.5	122.0	0.23	7.3	....	81.3	69.7	1.17
15	27.0	124.0	0.22	6.3	....	93.0	70.0	1.33
16	31.4	122.0	0.26	6.0	14.4	139.0	71.7	1.94
17	30.0	106.0	0.28	7.7	....	97.5	49.0	1.99
18	27.0	127.0	0.21	7.3	....	102.0	63.5	1.61
19	20.0	114.0	0.19	7.3	....	100.0	63.5	1.58
20	28.8	137.0	0.21	8.0	....	107.0	60.0	1.78
21	29.8	86.7	0.35	...	....	104.0	59.0	1.76
22	25.0	111.0	0.23	5.5	14.2	49.0	50.7	0.97
23	23.7	159.0	0.15	4.8	....	45.4	39.0	1.15
24	22.5	136.0	0.16	6.7	9.5	60.0	54.5	1.10
25	27.0	112.0	0.24	8.2	....	60.0	38.1	1.58
26	24.5	109.0	0.22	7.0	....	....	65.0	....
27	22.0	97.4	0.23	7.5	....	....	59.7	....
28	22.5	81.0	0.28	6.0	4.5	64.0	49.8	1.28

A number of interesting facts are brought out by this experiment. In the first place, the ammonia figures do not, in either case, indicate the relative ages of the cultures. The 10-day culture of *B. proteus*, for example, shows a lower concentration of ammonia nitrogen than the 8-day culture. Likewise, the 12-day culture of *B. pyocyaneus* contains a lower concentration of this compound than the 9-day culture. Many other instances of the same irregularity may be noted. It is apparent, therefore, that different cultures of the same organism on the same media and in exactly equal volumes may show quite different rates of ammonia-formation, even when made and grown under exactly the same conditions. In the case of both organisms, however, there is a general increase of ammonia-formation on the part of the older cultures up to the 15th or 16th day. After this the values fluctuate without any regular increase. Evidently, in the volume of media used, these organisms reach their maximum of ammonia-production in from about 14 to 18 days.

The amino-acid figures are comparable with those of Table 1. *B. proteus* gives a fluctuating, but consistently low value, while *B. pyocyaneus* gives a relatively high value, which shows a tendency to increase with the age of the culture. The ratios, when the volumes concerned are taken into account, show practically the same characteristics as those in Table 1.

An experiment similar to the foregoing was carried out also on the three organisms, *B. coli-communis*, *B. typhosus*, and *Sp. cholerae*. The technic was identical with that described in the case of *B. proteus* and *B. pyocyaneus*. Table 4 gives the data on this experiment.

TABLE 4

THE PRODUCTION OF AMINO-ACID AND AMMONIA BY BACTERIA IN A PEPTONE SOLUTION

Age of Cultures in Days	<i>B. Coli-Communis</i>			<i>B. Typhosus</i>			<i>Sp. Cholerae</i>		
	Amino-acid Nitrogen	Ammonia Nitrogen	Ratio: Amino-acid N	Amino-acid Nitrogen	Ammonia Nitrogen	Ratio: Amino-acid N	Amino-acid Nitrogen	Ammonia Nitrogen	Ratio: Amino-acid N
			Ammonia N			Ammonia N			Ammonia N
0	30.8	7.1	4.34	30.8	7.1	4.23	30.8	7.1	4.34
2	27.7	11.6	2.39	39.3	12.8	3.07	37.9	12.5	3.03
3	28.6	7.7	3.74	36.2	9.3	3.89	45.7	15.7	2.91
4	26.8	5.4	5.01	35.6	9.8	3.64	51.9	22.0	2.36
5	35.7	9.3	3.84	32.2	12.8	2.52	65.6	29.2	2.25
6	34.4	9.7	3.55	30.4	10.9	2.79	73.2	29.2	2.51
8	30.6	10.9	2.90	31.2	9.9	3.16	35.7	21.0	1.70
10	37.1	13.7	2.71	34.8	11.6	3.00	97.7	34.7	2.82
12	31.1	15.4	2.02	36.6	9.3	3.94	....	49.9	....
15	....	16.6	....	31.7	11.6	2.74	117.1	52.2	2.25
18	47.8	16.9	2.83	31.2	21.0	1.49	....	52.0	....
21	45.0	24.2	1.86	33.9	13.9	2.44	....	46.0	....

Little comment is necessary on these figures. The irregularity in the rate of ammonia-production which was mentioned in connection with Table 3 is found here also. It will be observed, however, that the figures representing the concentrations of amino-acid nitrogen deviate very little in the case of *B. coli* and *B. typhosus* from those found with uninoculated medium. In the culture of *Sp. cholerae* there is a decided increase of amino-acid nitrogen with the age of the culture. As would be expected, the ammonia-production by this organism is relatively high also.

With the same technic as that just described, cultures were examined of *B. proteus-vulgaris* on a 1% peptone solution containing 10 gm. of Liebig's meat extract per liter. In addition to amino-acid nitrogen and ammonia, both creatin and creatinin were determined in this case. Table 5, which gives the analytical data for the test, shows that on this medium also there is a very great irregularity in the production of ammonia. It is impossible to estimate from these data at what age cessation of chemical activity occurs. The creatinin values would indicate that this compound is continuously decomposed throughout the entire period of 37 days. The fluctuating values for creatin are undoubtedly due, in part, to the different degrees to which this substance is decomposed in sterilization.

It is evident from these examples that this method of procedure is unsuited to the quantitative study of the peptolytic activity of micro-organisms. Succeeding experiments were carried on, therefore, with

the technic employed in the original experiments; namely, that of inoculating a large volume of the medium and withdrawing samples day by day with a sterile pipet. It was further determined also to study the effect of glucose on amino-acid-production. It had been shown clearly by Kendall and his co-workers that this carbohydrate very considerably reduced the rate of ammonia-formation by most micro-organisms. It was a matter of interest to know also whether it would reduce amino-acid-production.

TABLE 5

THE PRODUCTION OF AMINO-ACID AND AMMONIA BY *B. PROTEUS-VULGARIS* IN A MEAT-EXTRACT PEPTONE SOLUTION

Age of Culture in Days	Amino-acid Nitrogen	Ammonia Nitrogen	Ratio:	Creatinin	Creatin	Amino-acid N plus Ammonia N
			Amino-acid N Ammonia N			
0	38.8	11.6	3.34	62.0	48.0	50.4
2	65.8	15.0	4.35	61.0	43.0	80.8
4	70.5	36.2	1.95	57.2	39.8	106.7
5	69.5	46.4	1.50	57.0	43.0	115.9
8	38.8	50.8	0.76	37.6	47.9	89.6
9	30.6	58.0	0.53	34.1	54.6	88.6
11	42.5	48.0	0.88	29.4	56.1	90.5
13	36.5	55.2	0.66	30.3	44.7	91.7
14	36.5	52.0	0.70	39.0	41.0	88.5
15	29.8	51.7	0.58	38.1	53.9	81.5
18	28.4	26.9	1.05	27.6	54.9	55.3
21	41.8	67.7	0.62	30.8	50.7	109.5
23	34.6	45.2	0.76	27.8	45.2	79.8
26	19.1	28.2	0.68	23.0	53.5	47.3
29	28.6	36.2	0.79	19.6	43.3	64.8
35	30.1	40.0	0.75	20.5	47.1	70.1
37	....	47.1	....	17.9	51.6	....

Seven organisms were investigated in this respect. The exact technic of the experiment was as follows. A large amount of a 2% peptone solution containing 0.5% NaCl was prepared and divided into 2 equal portions, to one of which was added approximately 1% of pure glucose. The media were then placed in 500-c.c. flasks, 300 c.c. to each. To each of the flasks containing glucose a small amount of  $\text{CaCO}_3$  was added. All the samples were sterilized together in the autoclave for 10 minutes under an extra pressure of 15 lb. They were all inoculated at 37 C. in the same incubator. The incubator was kept saturated with moisture to prevent evaporation. Samples, 15 c.c. in amount, were withdrawn at intervals as indicated in the tables, and were subjected at once to a half hour's heating in steam at 100 C. This would, of course, not sterilize the cultures of *B. subtilis*, but as the analyses were usually completed on the same day that the samples were taken (otherwise the samples were kept in the ice chest), it is not probable that any error was introduced by that fact.

Tables 6 to 12 inclusive give the results of the analyses of these samples. Charts 3 to 5 represent the same results in the form of curves.

An examination of the tables, or a glance at the curves, shows at once that, as would be expected, the peptolytic activity of *B. subtilis* is much greater than that of any of the rest, *Sp. metchnikovii* being the only one in this group which shows a comparable production of amino-acid or of ammonia. It is unfortunate that, as the result of a contamination on the 7th day, the examination of the only other organism having a gelatin-liquefying power, namely, *Staphylococcus pyogenes*, could not be carried out over the full period. The data obtained, however, indicate that in its chemical activity it is to be compared with the nonliquefying member of the group rather than with those mentioned.

The effect of glucose on the nitrogen metabolism of the cultures is apparent in both the amino-acid and the free-ammonia curves. In all cases, except those of *B. faecalis-alkaligenes* and *B. dysenteriae*, there is an unquestionably lower ammonia-production in the cultures containing glucose. The cultures of *B. dysenteriae*, when the slightly different conditions which may exist in the two flasks are taken into account, may be said to give practically identical ammonia curves. The same is probably true of *B. faecalis-alkaligenes*, altho it is interesting to note that, in this case, the curve of the glucose-containing culture is consistently above that of the one containing no glucose.

If it is assumed that neither of the organisms just mentioned is capable of utilizing glucose, it becomes difficult to explain the lower concentrations of amino-acids in the cultures containing this carbohydrate. There seem to be but two possible explanations of this fact. Either the glucose shows a protein-sparing action in that it decreases the peptone-decomposition by the organisms, or it acts in a manner that must be considered the direct opposite of this. That is, it brings about a more rapid breaking-down into simpler compounds of nitrogen of the amino-acids resulting from the splitting of the peptone. In this case it is necessary to assume that the breaking down of the amino-acids is not carried on to the free-ammonia stage or else that this stage is passed and free nitrogen or the oxids of nitrogen are formed.

The latter theory is so entirely in disagreement with all the well-known biologic reactions of glucose that it may safely be put out of consideration at once. If we accept then as an explanation of the facts the theory of decreased nitrogen metabolism, we have two instances of this phenomenon which are not indicated by the production of free ammonia.

TABLE 6

THE FORMATION OF AMINO-ACID AND AMMONIA FROM PEPTONE BY *B. PARATYPHOSUS*  
TOTAL NITROGEN = 304 MGM. PER 100 C.C.

Age of Culture in Days	Without Glucose			With Glucose		
	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio: Amino-acid N	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio: Amino-acid N
			Ammonia N			Ammonia N
0	42.4	3.5	12.10	42.4	3.5	12.10
1	56.7	4.3	13.20	34.1	2.0	17.10
2	42.5	4.4	9.67	38.6	...	.....
3	42.0	3.2	13.10	28.5	1.4	20.20
4	35.7	4.7	7.61	26.2	4.3	6.08
5	....	7.8	.....	34.0	3.0	11.30
6	36.3	...	.....	29.5	4.5	6.55
7	32.0	8.1	3.95	31.2	6.2	5.03
9	31.9	9.2	3.47	28.2	...	.....
11	33.1	9.0	3.67	35.4	2.4	14.80
14	41.0	9.9	4.14	34.2	4.1	8.35
18	42.7	9.1	4.68	39.2	2.0	19.70

TABLE 7

THE FORMATION OF AMINO-ACID AND AMMONIA FROM PEPTONE BY *B. ACIDI-LACTICI*  
TOTAL NITROGEN = 304 MGM. PER 100 C.C.

Age of Culture in Days	Without Glucose			With Glucose		
	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio: Amino-acid N	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio: Amino-acid N
			Ammonia N			Ammonia N
0	42.4	3.5	12.10	42.4	3.5	12.10
1	42.2	0.0	.....	38.3	0.0	.....
2	49.5	2.8	17.70	44.5	3.0	12.80
3	47.9	4.4	10.90	30.6	1.7	26.20
4	38.0	4.7	8.08	28.8	5.9	5.19
5	37.5	8.1	4.63	32.1	3.0	9.60
6	29.9	6.8	4.40	34.6	5.4	5.95
7	41.6	9.7	4.28	28.2	0.0	.....
9	34.5	12.2	2.83	36.8	...	.....
11	41.7	12.3	3.39	29.8	3.6	10.20
14	47.3	14.4	3.32	33.9	4.8	6.22
18	53.5	18.4	2.91	....	5.2	6.52

TABLE 8

THE FORMATION OF AMINO-ACID AND AMMONIA FROM PEPTONE BY *B. FECALIS-ALKALIGENES*  
TOTAL NITROGEN = 304 MGM. PER 100 C.C.

Age of Culture in Days	Without Glucose			With Glucose		
	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio: Amino-acid N	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio: Amino-acid N
			Ammonia N			Ammonia N
0	42.4	3.5	12.10	42.4	3.5	12.10
1	37.7	0.0	.....	28.6	3.5	8.17
2	52.8	2.6	20.30	36.1	3.5	10.30
3	34.9	4.6	7.59	33.8	4.9	6.91
4	25.0	5.1	4.90	32.6	5.7	5.72
5	30.0	5.9	5.08	25.0	5.8	4.32
6	31.5	7.0	4.50	25.2	7.7	4.42
7	35.8	6.4	5.59	19.0	12.2	1.56
9	36.8	6.1	6.04	27.5	6.5	4.23
11	38.3	5.3	7.23	23.0	5.1	4.51
14	38.9	3.4	11.40	28.0	5.1	5.49
18	33.0	5.2	6.35	26.5	4.9	5.42

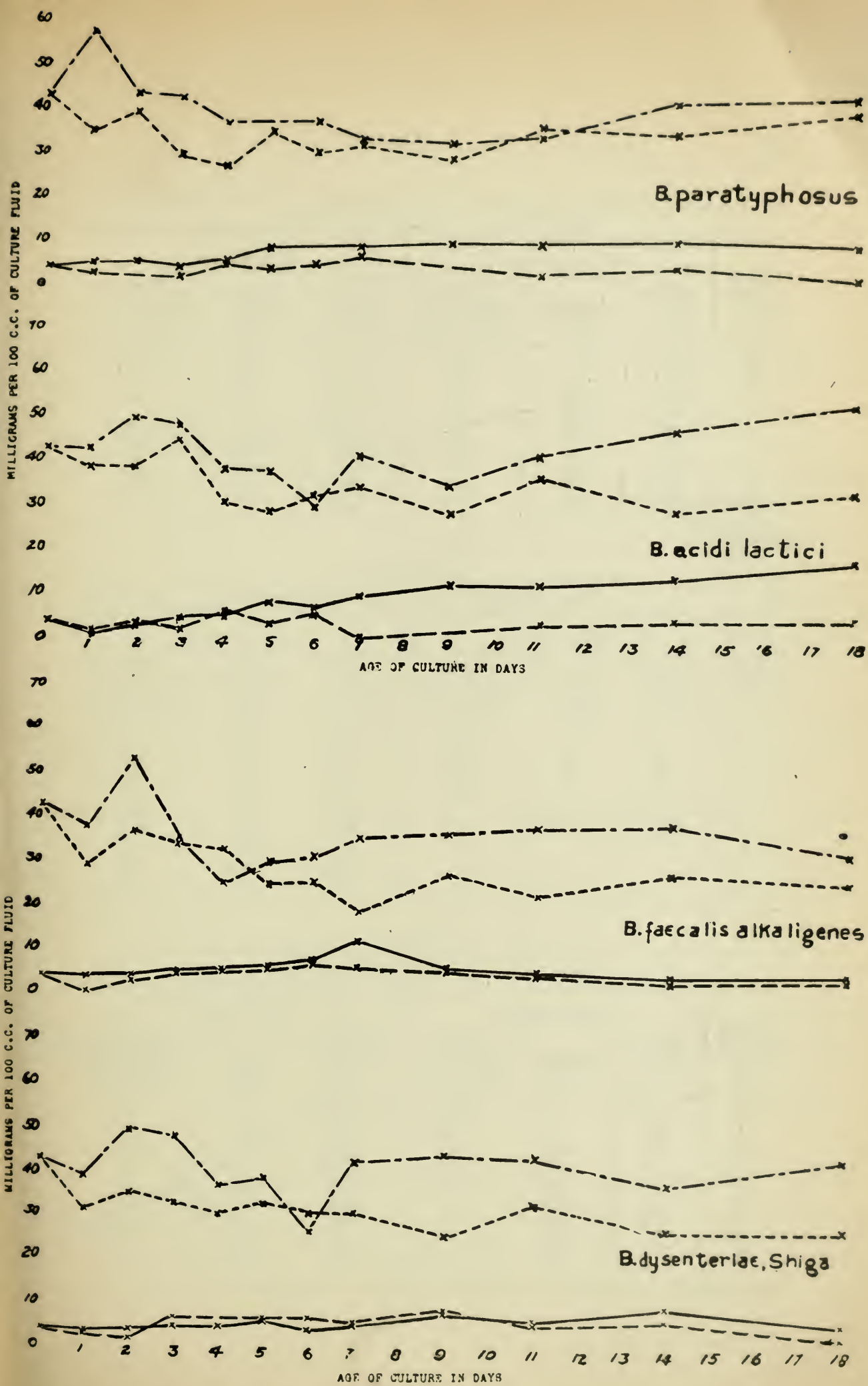


Chart 3. The formation by bacteria of amino-acid and ammonia from peptone.

TABLE 9

THE FORMATION OF AMINO-ACID AND AMMONIA FROM PEPTONE BY B. DYSENTERYÆ, SHIGA  
TOTAL NITROGEN = 304 MGM. PER 100 C.C.

Age of Culture in Days	Without Glucose			With Glucose		
	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio:	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio:
			Amino-acid N Ammonia N			Amino-acid N Ammonia N
0	42.4	3.5	12.1	42.4	3.5	12.1
1	38.3	3.2	12.0	30.6	2.6	14.2
2	49.1	3.8	12.9	34.9	1.5	33.0
3	47.9	3.8	12.6	32.8	7.6	4.3
4	36.6	4.7	7.8	30.2	...	....
5	38.7	6.2	6.2	32.9	6.1	5.4
6	26.5	4.2	6.3	30.8	6.6	4.7
7	42.9	5.1	8.4	31.0	5.4	5.7
9	44.3	7.9	5.6	25.8	8.8	2.9
11	43.6	6.5	6.7	33.2	5.5	6.0
14	37.9	9.7	3.9	27.4	6.3	4.4
18	43.4	5.9	7.4	27.5	2.6	10.6

TABLE 10

THE FORMATION OF AMINO-ACID AND AMMONIA FROM PEPTONE BY STAPHYLOCOCCUS PYOGENES  
TOTAL NITROGEN = 304 MGM. PER 100 C.C.

Age of Culture in Days	Without Glucose			With Glucose		
	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio:	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio:
			Amino-acid N Ammonia N			Amino-acid N Ammonia N
0	42.4	3.5	12.1	42.4	3.5	12.1
1	43.4	3.6	12.0	37.5	3.2	11.7
2	43.2	5.2	8.3	42.9	1.2	36.6
3	50.7	4.9	10.3	43.5	3.5	12.4
4	39.3	8.5	4.6	35.9	4.1	8.8
5	46.2	7.3	6.3	40.6	2.8	14.5
6	31.8	...	....	45.5	4.5	10.1
7	42.0	7.7	5.5	41.0	10.2	40.2
8	....	...	....	47.3	6.1	7.8
11	....	...	....	47.8	6.0	8.0
14	....	...	....	56.2	5.5	10.2
18	....	...	....	62.5	5.2	12.0

TABLE 11

THE FORMATION OF AMINO-ACID AND AMMONIA FROM PEPTONE BY SP. METCHNIKOVII  
TOTAL NITROGEN = 304 MGM. PER 100 C.C.

Age of Culture in Days	Without Glucose			With Glucose		
	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio:	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio:
			Amino-acid N Ammonia N			Amino-acid N Ammonia N
0	42.4	3.5	12.1	42.4	3.5	12.1
1	37.3	2.2	16.9	40.3	0.0	....
2	50.9	6.5	7.8	39.1	1.2	32.6
3	51.4	11.9	4.3	43.4	10.1	4.3
4	56.9	15.3	3.7	41.2	9.3	4.4
5	49.5	21.7	2.3	40.0	11.5	3.5
6	55.5	29.8	1.8	43.9	11.1	4.0
7	42.0	55.2	1.7	62.2	10.8	5.8
9	75.9	27.0	2.7	40.0	11.7	3.4
11	92.4	50.5	1.8	48.5	9.6	5.1
14	91.7	41.6	2.2	52.8	11.3	4.7
18	113.2	46.6	2.4	67.8	14.5	4.7

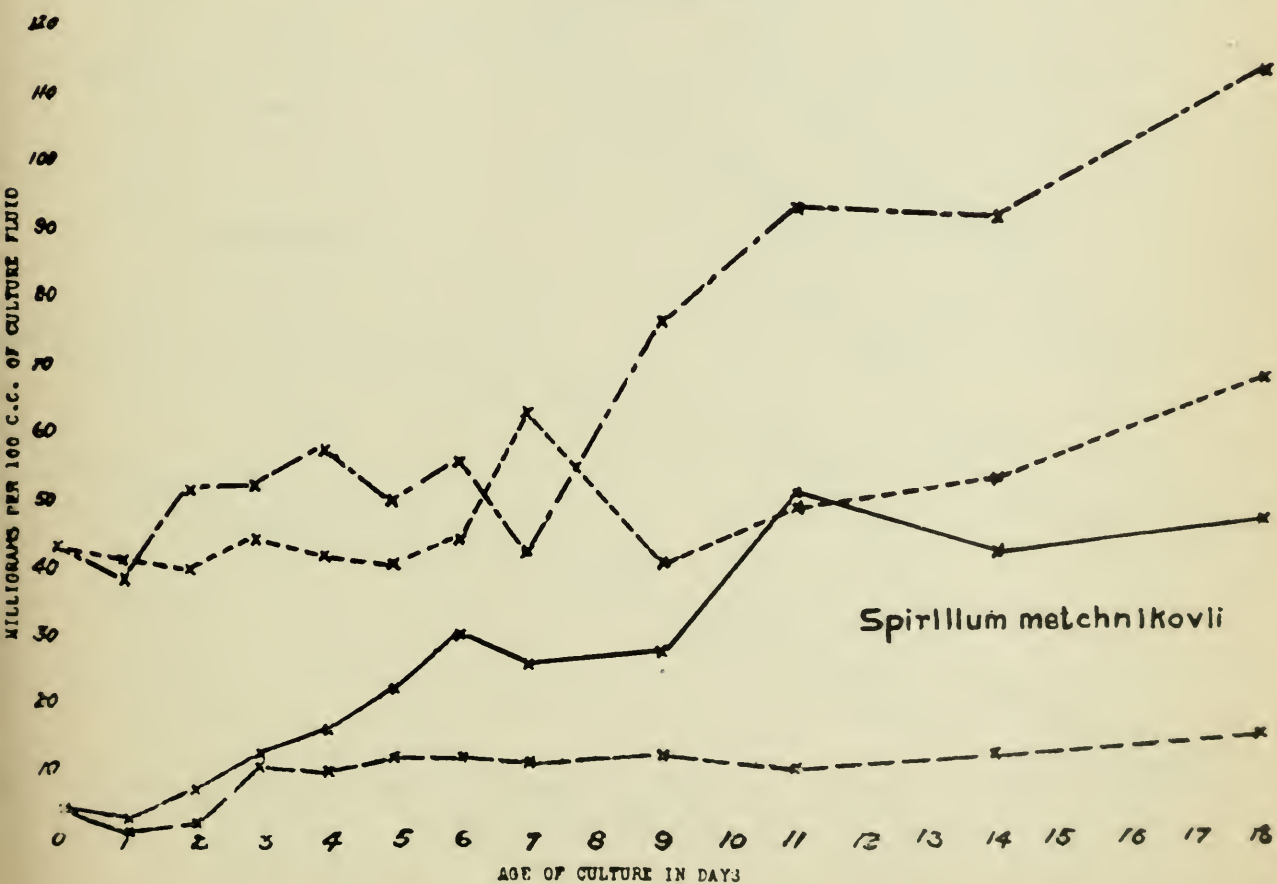
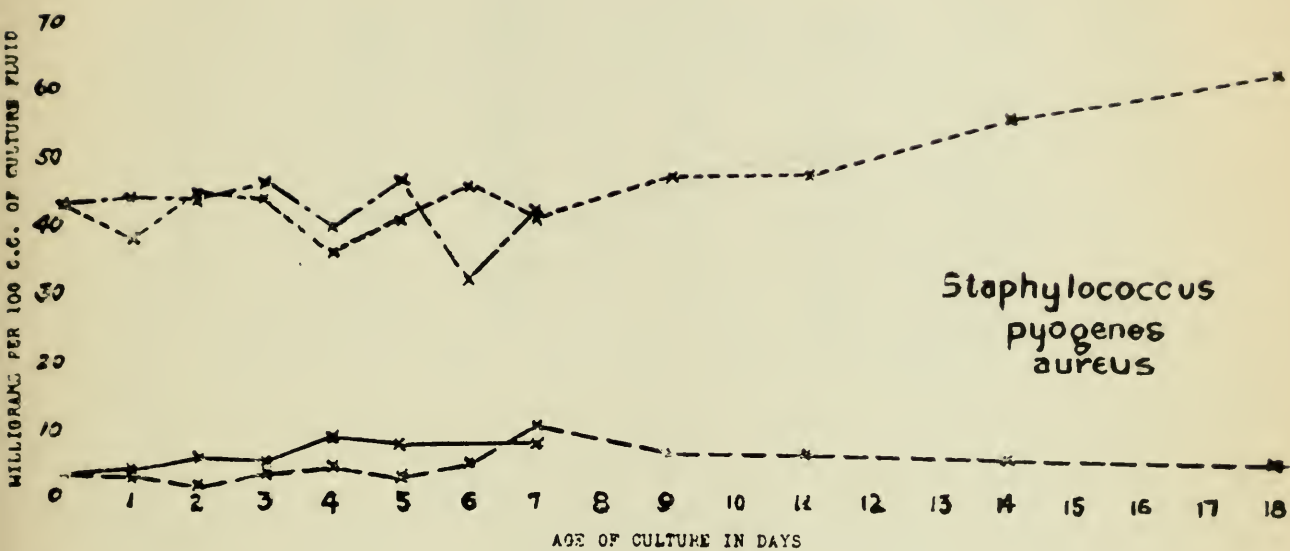


Chart 4. The formation by bacteria of amino-acid and ammonia from peptone.

TABLE 12  
THE FORMATION OF AMINO-ACID AND AMMONIA FROM PEPTONE BY *B. SUBTILIS*  
TOTAL NITROGEN = 304 MGM. PER 100 C.C.

Age of Culture in Days	Without Glucose			With Glucose		
	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio: Amino-acid N	Amino-acid Nitro-gen	Ammonia Nitro-gen	Ratio: Amino-acid N
			Ammonia N			Ammonia N
0	42.4	3.5	12.1	42.4	3.5	12.1
1	44.1	2.4	18.4	29.2	2.0	14.6
2	60.1	13.9	4.3	42.1	4.3	9.8
3	79.4	19.4	4.1	51.3	4.4	11.7
4	86.8	29.4	2.9	50.2	8.5	5.9
5	94.0	35.9	2.6	65.8	15.4	4.3
6	100.0	43.1	2.0	71.0	20.1	3.5
7	122.0	53.1	2.3	92.3	22.7	4.1
9	117.5	55.5	2.1	97.8	31.1	3.1
11	114.0	62.5	1.8	129.3	35.0	3.7
14	101.2	68.7	1.5	112.0	37.8	3.0
18	109.0	65.9	1.7	135.8	51.2	2.6

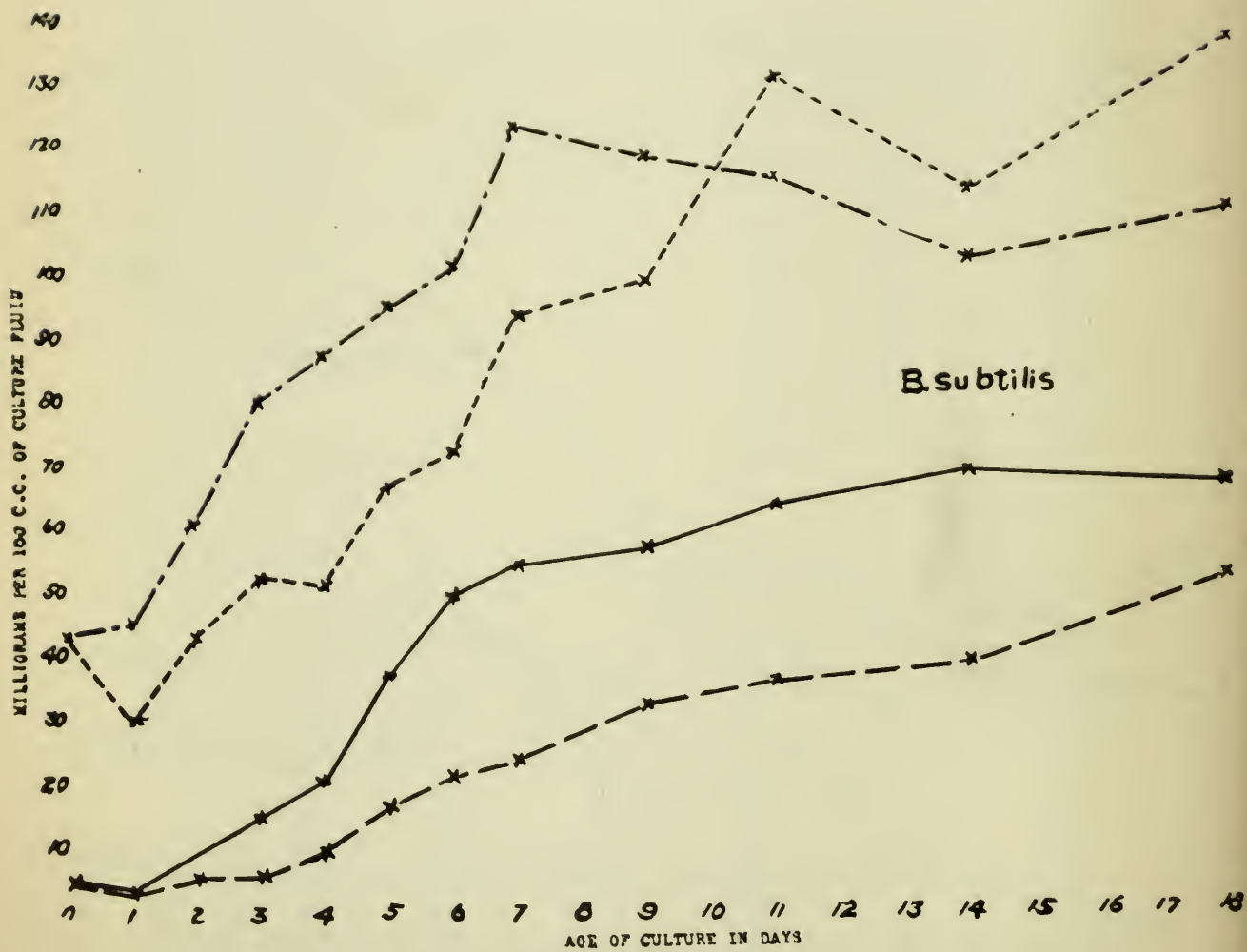


Chart 5. The formation by bacteria of amino-acid and ammonia from peptone.

That the presence of glucose might decrease the peptone-splitting power of the cultures by preventing, to some extent, the reproduction of the organisms is contrary to experience, and would furthermore make the identical ammonia curves difficult to explain.

The decrease during the first 24-48 hours in the amount of free ammonia which was originally present in the culture medium is noticed in the case of all the organisms studied. This decrease seems to take place to practically the same extent in the cultures containing glucose. It is undoubtedly true that in such cultures the most rapid multiplication of the organisms takes place during the first 24-48 hours. It is therefore probable that this ammonia is utilized for the synthesis of bacterial protein. That ammonium salts are so utilized by certain micro-organisms when these salts are their only source of nitrogen is a well-established fact. But that such micro-organisms as those investigated should utilize ammonia in the presence of other forms of nitrogen seems at first a little surprising. A glance at the curves representing the concentrations of ammonia in the glucose-containing culture will show, however, that this compound is continuously utilized by the bacteria in question. Frequent decreases in concentration take place with all but the strongly proteolytic organism, *B. subtilis*. That the same decreases are not observed in the cultures not containing glucose is probably due to the greater proteolysis in the latter rather than to an increased assimilation of ammonia in the presence of the sugar. It is safe to assume that in no case does the ammonia found represent the entire amount formed by the organisms in the course of metabolism.

Also, an initial lowering of the amino-acid concentrations is observed in most of the cultures. This lowering is generally greater in the glucose-containing cultures, a fact which is doubtless explained by the decreased proteolytic activity in the latter.

In the cultures of *B. subtilis* and *Sp. metchnikovii* there is a tendency toward accumulation of amino-acids, both in the presence and in the absence of glucose. In the case of *B. subtilis* this accumulation takes place after the first 24 hours at about an equal rate in both cultures until the 7th day is reached. After this the culture not containing the sugar shows a decrease while the one containing the sugar continues to show an increase at about the same rate. The maximal concentration of amino-acid in the case of the latter is not passed at the end of the experiment on the 18th day.

The ammonia curves of this organism are roughly parallel to the amino-acid curves. The sum of the amino-acid nitrogen and ammonia

nitrogen in the non-glucose-containing culture on the 7th day is nearly the same as the sum of these two substances in the glucose-containing culture on the 18th day, and is approximately equal to  $\frac{3}{5}$  of the total nitrogen of the culture medium. Of all the cultures tested, that of *Sp. metchnikovii* shows the greatest reduction of proteolytic activity due to the presence of glucose. In the sugar-containing culture of this organism the concentration of ammonia is uniformly low while in that free of sugar it reaches a value of 50.5 mgm., or about 17% of the total nitrogen of the medium. The amino-acid content of this culture attains the value of 113.2 mgm., or about 37% of the total nitrogen content. Taking into account, as will be pointed out later, the presence of other simple compounds of nitrogen, we can say that this organism in the absence of sugar is capable of decomposing almost completely a 2% solution of peptone. By the same reasoning *B. subtilis* may be said to be capable of this even in the presence of 1% of glucose.

#### THE PRODUCTION OF AMINO-ACID AND AMMONIA BY BACTERIA IN MEDIA CONTAINING GELATIN

The medium used in these tests was a 1% peptone solution containing 0.5% of NaCl to which was added 5% of pure gelatin. The medium was cleared by means of egg white, made neutral to phenolphthalein, and filtered until clear. To one half of the solution so prepared 1% of glucose was added. Both portions were then placed in 300-c.c. flasks, 200 c.c. to each. Calcium carbonate was added to the samples containing the carbohydrate. All the flasks were sterilized under 15 pounds' extra pressure for 10 minutes. They were then all inoculated at the same time and placed together in the same incubator at 37 C.

All the organisms used were capable of liquefying gelatin except *B. faecalis-alkaligenes* and *B. cloacae*. The strict anaerobe, *B. aerogenes-capsulatus*, and the facultative anaerobe, *B. pyocyaneus*, were grown under anaerobic conditions by keeping the surface of the medium covered with a thick layer of sterilized paraffin oil. Samples were removed at definite intervals by means of sterile pipets. These samples were heated in steam at 100 C. for one-half hour and at once subjected to analysis.

Tables 13 to 16 inclusive give the analytical results. The total nitrogen of the sterile medium was 828.6 mgm. per 100 c.c. *B. subtilis* shows much the same characteristics in its action on gelatin as on the peptone solution. It produces both amino-acid and free ammonia much more rapidly at first in the absence of glucose, but the rate of the production of these substances in the presence of the sugar increases in the later stages until, at the end of the 5th week, the concentration of ammonia nitrogen is actually greater in this culture than in the culture containing no glucose and the concentration of the amino-acid nitrogen is nearly equal to that in the culture not containing glucose.

TABLE 13  
THE PRODUCTION OF AMINO-ACID AND AMMONIA FROM GELATIN BY BACTERIA  
TOTAL NITROGEN = 828.6 MGM. PER 100 C.C.

Age in Days	B. Subtilis				B. Cloacae			
	Without Glucose		With Glucose		Without Glucose		With Glucose	
	Amino- acid N	Am- monia N	Amino- acid N	Am- monia N	Amino- acid N	Am- monia N	Amino- acid N	Am- monia N
0	42.5	5.9	42.5	5.9	42.5	5.9	42.5	5.9
1	.....	.....	.....	.....	58.0	0.0	45.7	1.3
2	79.2	11.4	65.0	5.2	45.8	0.0	38.2	0.0
4	130.7	20.5	97.3	5.9	69.3	5.4	52.3	0.0
7	206.8	65.2	120.4	0.0	71.8	9.2	63.3	0.0
10	225.7	88.0	140.4	14.9	56.9	22.2	44.8	9.1
15	225.1	73.5	189.0	39.0	63.7	33.3	50.8	27.2
24	185.0	136.1	185.0	113.0	62.8	36.7	32.6	24.0
34	172.0	133.4	159.5	164.3	65.5	43.5	56.5	50.7

TABLE 14  
THE PRODUCTION OF AMINO-ACID AND AMMONIA FROM GELATIN BY BACTERIA  
TOTAL NITROGEN = 828.6 MGM. PER 100 C.C.

Age in Days	B. Faecalis-Alkaligenes				B. Pyocyaneus, Aerobic			
	Without Glucose		With Glucose		Without Glucose		With Glucose	
	Amino- acid N	Am- monia N	Amino- acid N	Am- monia N	Amino- acid N	Am- monia N	Amino- acid N	Am- monia N
0	42.5	5.9	42.5	5.9	42.5	5.9	42.5	5.9
1	51.8	0.0	....	....	.....	1.3	44.3	0.0
2	50.5	0.0	85.5	0.0	50.2	0.0	44.3	0.0
4	54.7	0.0	45.0	0.0	47.4	2.8	54.7	0.0
7	36.4	0.0	38.8	0.0	87.6	11.5	48.7	0.0
10	55.4	2.3	42.0	2.8	109.5	21.9	52.3	0.0
15	39.9	7.0	38.2	6.5	279.0	34.2	55.0	1.0
24	63.7	14.3	42.6	12.1	145.0	49.7	113.0	18.6
34	62.0	14.7	46.8	18.1	161.0	41.2	202.4	35.5

TABLE 15  
THE PRODUCTION OF AMINO-ACID AND AMMONIA FROM GELATIN BY BACTERIA  
TOTAL NITROGEN = 828.6 MGM. PER 100 C.C.

Age in Days	B. Pyocyaneus, Anaerobic				B. Welchii			
	Without Glucose		With Glucose		Without Glucose		With Glucose	
	Amino- acid N	Am- monia N	Amino- acid N	Am- monia N	Amino- acid N	Am- monia N	Amino- acid N	Am- monia N
0	42.5	5.9	42.5	5.9	42.5	5.9	42.5	5.9
1	.....	0.0	.....	....	.....	0.0	.....	....
2	55.4	.....	48.6	0.0	47.1	0.0	49.3	0.0
4	48.7	0.0	59.7	0.0	79.1	0.0	186.4	19.9
7	56.0	0.0	53.1	0.0	36.4	0.0	260.7	76.6
10	67.3	5.7	34.4	2.8	56.3	0.0	277.2	92.2
15	94.0	13.9	99.0	9.3	51.3	1.3	305.0	95.2
24	159.1	34.7	105.3	17.8	66.2	3.2	303.0	85.7
34	194.2	40.3	137.9	25.9	53.7	3.6	292.0	30.3

TABLE 16

THE PRODUCTION OF AMINO-ACID AND AMMONIA FROM GELATIN BY BACTERIA  
TOTAL NITROGEN = 828.6 MGM. PER 100 C.C.

Age in Days	Sp. Cholerae			
	Without Glucose		With Glucose	
	Amino-acid N	Ammonia N	Amino-acid N	Ammonia N
0.....	42.5	5.9	42.5	5.9
1.....	48.0	0.0	....	...
2.....	58.0	3.9	49.5	0.0
4.....	48.7	0.0	70.5	0.0
7.....	89.7	1.76	40.7	0.0
10.....	123.8	30.4	42.4	0.2
15.....	174.0	36.8	41.8	4.2
24.....	237.0	78.1	40.3	8.3
34.....	265.3	100.7	45.1	4.1

The initial decrease in free ammonia is not observed in the sugar-free culture, probably because of the fact that a sample was not analyzed at the end of one day's incubation. This decrease is apparent, however, in the glucose-containing culture, and the latter also shows evidence of the continuous utilization of free ammonia in the falling off of the concentration from 5.9 mgm. per 100 c.c. on the 4th day to 0 on the 7th. Both the ammonia- and the amino-acid-production reach higher values than in the corresponding cultures on peptone alone. The sum of the two forms of nitrogen in the sugar-free culture does not differ greatly, on the last day of the experiment, from that in the case of the sugar-containing cultures, the two sums being approximately 37% and 39% respectively of the total nitrogen of the sterile medium.

The spirillum of Asiatic cholera offers a very interesting example of the protein-sparing action of glucose. In the concentrations of both amino-acid and free-ammonia the differences between glucose and nonglucose cultures are enormous. The data show that in the absence of the carbohydrates the proteolytic activity of this organism is considerable, as great, in fact, as that of *B. subtilis* or *B. pyocyaneus*. With glucose present, however, the indication is that the gelatin is attacked very little, if at all, since the figures are much lower even than those shown by this organism on a pure peptone medium (Table 3). It is evident that the effect of the sugar is continued throughout the entire time of the experiment. That chemical activity was not brought to a standstill by products of sugar-decomposition is evident from the fluctuating values of ammonia and amino-acid nitrogen.

The data for *B. cloacae* and for *B. faecalis-alkaligenes* indicate that these two organisms are comparable with respect to their activity in the gelatin medium. The amino-acid concentrations are low in both cases. *B. cloacae*, however, forms comparatively large amounts of free ammonia, the rate of formation being slightly higher in the sugar-free culture. The amino-acid figures do not differ greatly in the two cultures of this organism. Considering the well-known utilization of glucose by *B. cloacae*, it is surprising that its protein-sparing action is so little marked in this case. *B. faecalis-alkaligenes* lives up to its reputation in these cultures, also, in showing no decrease in its ammonia-production in the presence of glucose.

The two sets of cultures of *B. pyocyaneus*, the one grown under aerobic, the other under anaerobic conditions, furnish an instructive comparative study in the biochemistry of micro-organisms. The surprising fact in these data is that so little difference is shown between the two sets. On the whole the greater chemical activity seems to be shown in the cultures grown without the exclusion of oxygen. This difference is apparent, however, only during the first half of the period of the experiment. The effect of glucose is greater in the aerobic than in the anaerobic cultures. As multiplication appeared to be much slower in the latter, it is very likely that the differences which do exist are to be ascribed to differences in the numbers of organisms rather than to actual differences in the course or extent of chemical change due to the presence or absence of oxygen. Likewise, the apparently lowered protein-sparing effect of glucose in the anaerobic cultures is probably due to a difference between the numbers of organisms, for there was an unquestionably heavier growth in the anaerobic culture containing the sugar than in the sugar-free culture grown under the same conditions.

The cultures of *B. welchii* show this effect of glucose very plainly. The enormously greater chemical activity of this organism when grown with glucose can only be attributed to greatly increased multiplication in the presence of glucose. The appearance of the cultures fully substantiates this conclusion.

It will be observed from the data that the proteolytic activity of *B. welchii* when grown under favorable conditions is considerable. As will be pointed out later, the nitrogen determined in the form of amino-acid and ammonia cannot be assumed to represent nearly all the nitrogen which is removed from its combinations in the protein molecule to form simpler compounds. When we consider, therefore, that

on the 15th day of incubation the nitrogen so determined, amounts to nearly 50% of the total nitrogen of the medium, we can scarcely agree with Rettger's<sup>40</sup> observation that this bacillus is primarily a fermenting organism.

The initial reduction in amount of the free ammonia which is originally present in the medium occurs in all of the cultures except the sugar-free culture of *B. subtilis*. The probable reason for this exception has already been suggested. In all cases this initial reduction takes place until the ammonia value actually reaches zero, and thereafter, in a number of the cultures, this compound remains absent for several days. This peculiar behavior was not shown by the pure peptone cultures and therefore it must be ascribed to the presence of gelatin. Obviously, the amino-acids resulting from the decomposition of this protein are broken down to the form of ammonia to a much smaller extent than those resulting from the splitting of Witte's peptone. Probably in the presence of an abundance of nourishment the decomposition is not carried so far.

EVIDENCE OF THE EXISTENCE OF CONSIDERABLE QUANTITIES OF  
NITROGEN IN COMPOUNDS INTERMEDIARY BETWEEN AMINO-  
ACID AND AMMONIA

Investigators who have speculated on the manner in which the amino-acids resulting from protein-decomposition by micro-organisms are further broken down in the course of putrefaction have taught us to suppose that the first reaction to take place is deaminization with the splitting off of ammonia.<sup>41</sup> This would mean, of course, that all the nitrogen of that group determined by the Van Slyke method, i. e., the alpha amino nitrogen, is transformed into ammonia. That this is not true in the case of the cultures investigated by us becomes evident when the tables are studied more carefully. Every decrease in amino-acid content which occurs in the interval between two analyses must represent, of course, the minimal amount of this form of nitrogen which has been altered during the period. If such alteration consisted in large part or entirely in deaminization, then the decrease should appear as a corresponding increase in ammonia nitrogen. The instances in which this is true in the data given are so rare as to be considered purely accidental. That this extra amount of amino-acid nitrogen metabolized could be to any considerable degree accounted for by ammonia lost through volatilization is made improbable from the following experiment: Five cubic centimeters of a 2% peptone

<sup>40</sup> Jour. Biol. Chem., 1908, 4, p. 45.

<sup>41</sup> Lafar: Handbuch der technischen Mykologie, 1904-1906, 3, p. 103.

solution were placed in each of 6 tubes, sterilized, and inoculated severally with the organisms as indicated in Table 17. After 15 days' incubation at 37 C. the tubes were again sterilized and the whole contents of each submitted to a total nitrogen-determination. The results are given in Table 17. The figures represent the total nitrogen in milligrams in the 5 c.c. of the corresponding culture.

TABLE 17  
NITROGEN LOST FROM BACTERIAL CULTURES BY VOLATILIZATION

Organism	Milligrams of Total Nitrogen in 5 c.c. of Culture Medium After 15 Days' Incubation
Sterile control.....	15.95
<i>B. coli-communis</i> .....	15.64
<i>B. typhosus</i> .....	15.94
<i>B. pyocyaneus</i> .....	15.61
<i>B. faecalis-alkaligenes</i> .....	15.72
<i>B. subtilis</i> .....	14.39

It is seen that *B. subtilis* is the only one of the organisms investigated in this respect which lost any appreciable quantity of nitrogen during the 15 days' incubation. Even its loss could by no means account for the differences mentioned. Berghaus<sup>42</sup> also gives data on the loss of ammonia through volatilization. Tho ammonia is undoubtedly utilized to a certain extent by most of the species studied, the amount utilized must necessarily be small. That part of it which is used for the synthesis of bacterial protein or the metabolism of which results in compounds which are not volatile could not be great enough to be considered in the present connection.

The only conclusion which can be drawn from the facts in the case, therefore, is that the greater part of the reported losses in amino-acid nitrogen which take place from time to time is due to the conversion of this nitrogen into compounds other than ammonia. Further, the continued low concentration or the entire absence of the latter, as seen particularly in the gelatin cultures, during the first days of the experiments, points to the conclusion that ammonia arises from the decomposition of these other compounds, and that the latter are therefore to be regarded as important stages in the complete decomposition of protein. That some of them are true end products, and undergo no further change through the action of the micro-organism concerned is evidenced by the slight extent to which creatin is attacked by *B. proteus* (Table 5), which, as seen in Table 1, is capable of forming this substance from peptone.

<sup>42</sup> Arch. f. Hyg., 1907, 64, p. 1.

THE FORMATION BY BACTERIA OF UREA, URIC ACID, ALLANTOIN,  
CREATIN, AND CREATININ

*Urea and Uric Acid.*—All the peptone cultures described thus far in this paper were examined in several stages of their growth for urea and uric acid. Neither of these compounds was found in any case.

The urease method originated by Van Slyke<sup>43</sup> was used for the detection of the former, and the colorimetric method used by Folin and Denis,<sup>44</sup> for the latter. Both these methods were proved to be efficient by determinations made on cultures to which weighed amounts of the pure chemicals had been added. The medium used in the case of uric acid was a solution containing 10 gm. asparagin, 2.5 gm. Na<sub>2</sub>CO<sub>3</sub>, 2 gm. Na<sub>2</sub>HPO<sub>4</sub>, traces of MgSO<sub>4</sub> and CaCl<sub>2</sub> and 0.902 gm. of uric acid per liter. The time of incubation was 15 days. The medium used in the case of urea was a 2% peptone solution containing about 10 gm. of urea per liter. The cultures were incubated for 7 days.

TABLE 18  
DECOMPOSITION OF URIC ACID BY BACTERIA

Organism	Ammonia Nitro- gen per 100 c.c. Culture Medium	Amino-acid Nitro- gen per 100 c.c. Culture Medium	Uric Acid per 100 c.c. Culture Medium
Sterile control.....	5.3 mgm.	94.5 mgm.	65.7 mgm.
B. coli-communis.....	112.0 mgm.	36.0 mgm.	7.5 mgm.
B. acidi-lactici.....	102.6 mgm.	7.3 mgm.	None
B. pyocyaneus.....	39.1 mgm.	22.5 mgm.	None
B. smegmae.....	43.2 mgm.	68.0 mgm.	None
Sp. cholerae.....	41.5 mgm.	32.6 mgm.	None

TABLE 19  
DECOMPOSITION OF UREA BY BACTERIA\*

Organism	Ammonia Nitrogen in 5 c.c. Culture Fluid	Urea Nitrogen in 5 c.c. Culture Fluid	Nitrogen of Urea Decomposed in 5 c.c. Culture Fluid
Sterile control.....	None	22.28 mgm.	None
Staph. aureus.....	3.03 mgm.	21.75 mgm.	0.53 mgm.
B. coli-communis.....	3.12 mgm.	21.23 mgm.	1.05 mgm.
B. acidi-lactici.....	2.31 mgm.	15.48 mgm.	6.80 mgm.
B. faecalis-alkaligenes.....	0.52 mgm.	6.73 mgm.	15.45 mgm.
B. paratyphosus.....	2.24 mgm.	6.37 mgm.	15.81 mgm.

\* In this test inoculations were made into 5-c.c. portions of the culture medium, and the tubes were incubated for 7 days.

It is not surprising that uric acid and urea are not to be found in bacterial cultures when it is considered how easily and completely both are decomposed by bacteria. Tables 18 and 19 give the results of brief tests designed to show this.

*Allantoin.*—Numerous attempts were made to prove the presence of allantoin in cultures on pure peptone solutions as well as in those on peptone solutions containing uric acid, but it was impossible to find

<sup>43</sup> Van Slyke and Cullen: Proc. Soc. Exper. Biol. and Med., 1913, 11, p. 56.

<sup>44</sup> Jour. Biol. Chem., 1913, 14, p. 95.

a method by which all interfering substances were excluded. The Wiechowski<sup>45</sup> method, for which great accuracy is claimed in the determination of allantoin in urine, was employed repeatedly, but without success.

This method consists in precipitation by phosphotungstic acid, the removal of the excess of this reagent by means of lead oxid and acetic acid, the elimination of the chlorid with silver acetate, and the final precipitation of all heavy metals with hydrogen sulfid. After removal of the latter by aeration the solution is made alkaline with magnesium oxid and the allantoin precipitated by means of a solution consisting of 20% sodium acetate and 1% mercuric acetate.

It was found that in all bacterial cultures on peptone media a precipitate was obtained at this point which had a varying, in some cases high, total-nitrogen content. Moreover, the sterile control usually gave a precipitate here also, which varied from a slight opalescence to an appreciable deposit. It was not possible to prepare the characteristic crystals of allantoin from any of these precipitates. It is probable that the latter consisted, in the most part, of amino-acids which escaped precipitation by the phosphotungstic acid. Levene and Beatty<sup>46</sup> showed that many of these acids are precipitated completely by this reagent only when it is present in very high concentration. Such concentrations would be entirely impractical in the present connection.

Attempts to show that uric acid is decomposed by bacteria with the formation of allantoin were unsuccessful for the same reasons as have just been outlined. A medium having asparagin as the nitrogenous base was also used in these experiments. The Wiechowski method was inapplicable here also, since asparagin, not being precipitated by phosphotungstic acid, is brought down with the allantoin by the sodium-acetate mercuric-acetate reagent. If allantoin was ever present in this precipitate, it was there in such small quantities that it could not be detected in the presence of asparagin.

This inability to separate allantoin from asparagin would seem to render the Wiechowski method inapplicable also to the determination of allantoin in plants. In fact, it has been shown recently in this laboratory that a very large portion of the nitrogen determined as allantoin in plant tissue by this method is in reality asparagin, where this substance is present in considerable amounts.\*

The problem of the formation of allantoin by bacteria seems to depend for its solution on the perfection of the methods for the determination of this compound and its separation from interfering substances.

*Creatin and Creatinin.*—Most of the peptone cultures or peptone solutions reported in the first part of this paper were tested for creatin and creatinin. In general, the results were in close agreement with those of Fitzgerald and Schmidt.<sup>22</sup> That is, only *B. proteus* was found to form appreciable amounts of creatinin on solutions of peptone alone. It was found however that in those cultures containing glucose, a test for this substance and for creatin gave positive results in a large num-

<sup>45</sup> Neubauer's *Analyse des Harns*, 1913, 2, p. 1076.

<sup>46</sup> Levene and Beatty, *Ztschr. f. physiol. Chem.*, 1906, 47, p. 149.

\* Yet unpublished thesis of G. C. Swan, Stanford University, May, 1915.

ber of cases. More careful investigation of this subject was considered important, and the following experiment was therefore carried out:

A large amount of 2% peptone solution containing 0.5% NaCl was divided in half and to one portion 1% glucose was added. The two portions were placed in sterile flasks, 100 c.c. to each, and sterilized in the autoclave for 10 minutes. The flasks were then inoculated and placed in the incubator at 37 C. Each organism to be tested was inoculated into a flask containing glucose and into one not containing glucose.

Folin<sup>47</sup> claimed accuracy for his methods for the determination of creatin and creatinin in the presence of considerable quantities of glucose. Nevertheless, it was considered essential to maintain two sterile controls, one with, and one without, glucose. Samples were withdrawn from each of the cultures and from the controls at definite intervals and after sterilization in steam at 100 C., they were subjected to analysis for creatinin and creatin, by Folin's methods.

Tables 20 and 21 give the results of these analyses. The values are in milligrams per 100 c.c. The fact that neither of the sterile controls gave at any time a color reaction seems sufficient to establish at once the noninterference of glucose with the determinations. We may assume, therefore, that the figures given indicate with reasonable accuracy the comparative creatinin- and creatin-forming power of the micro-organisms in question.

It will be observed that only *B. proteus* and *Sp. cholerae* form both creatin and creatinin in the absence of sugar. *B. subtilis* forms creatin but not creatinin. Of the cultures on the glucose-containing solutions, only *B. faecalis-alkaligenes* fails to give the reaction for creatinin. This culture shows on the second day a considerable quantity of creatin. Tests for this substance thereafter, however, are all negative.

Examination of the tables will suggest that there are several possibilities for the differentiation of species by this method. For example, on the second day the amount of creatinin in the culture of *B. typhosus* is 5.9 mgm. per 100 c.c., an amount which is easily detectable in a 5-c.c. portion. The corresponding cultures of *B. coli* and *B. faecalis-alkaligenes* both are negative in the test for creatinin. Likewise, *Sp. cholerae* gives 6.6 mgm. of creatinin on the second day, while the morphologically and culturally similar organism, *Sp. metchnikovii*, gives only a trace. It is interesting to note that in the point of their creatin-production all five of the organisms mentioned, show just the reverse characteristics; the cultures of *B. coli*, *B. faecalis-alkaligenes*, and *Sp. metchnikovii* show high concentration, while those of *B. typhosus* and *Sp. cholerae* give low values for this compound.

In most of the cultures the maximal concentration of both creatin and creatinin seemed to be reached from about the 5th to the 8th days.

<sup>47</sup> Jour. Biol. Chem., 1914, 17, p. 475.

After that the amount seemed to suffer some decrease. In the case of *B. pyocyaneus* and *B. subtilis* a maximum is not shown in the time of the experiment. The creatinin content in the culture of *Staph. pyogenes* shows a continuous increase, but its creatin content falls nearly to zero after the second day.

TABLE 20  
FORMATION OF CREATININ IN PEPTONE CULTURES OF MICRO-ORGANISMS

Culture	2 Days Old		5 Days Old		8 Days Old		14 Days Old	
	1*	2*	1	2	1	2	1	2
<i>B. proteus-vulgaris</i> .....	—	3.4	Trace	4.5	2.6	5.1	4.6	5.8
<i>B. pyocyaneus</i> .....	—	4.0	—	6.3	—	9.4	—	23.1
<i>B. typhosus</i> .....	—	5.9	—	8.0	—	6.8	—	6.9
<i>B. coli-communis</i> .....	—	—	—	5.2	—	3.2	—	Trace
<i>Sp. cholerae</i> .....	—	6.6	—	8.8	2.9	10.4	—	Trace
<i>B. subtilis</i> .....	—	—	—	5.6	—	14.6	—	25.0
<i>B. paratyphosus</i> .....	—	5.1	—	7.3	—	5.9	—	6.1
<i>B. cloacae</i> .....	—	3.9	—	4.4	—	4.7	—	—
<i>B. faecalis-alkaligenes</i> .....	—	—	—	—	—	—	—	—
<i>Sp. metchnikovii</i> .....	—	Trace	—	Trace	—	4.7	—	7.3
<i>Staph. pyogenes</i> .....	—	Trace	—	3.5	—	5.0	—	11.6
<i>B. dysenteriae, Shiga</i> .....	—	3.8	—	4.1	—	5.4	—	5.7
<i>B. acidi-lactici</i> .....	—	3.7	—	4.2	—	5.0	—	5.1
<i>B. prodigiosus</i> .....	—	Trace	—	3.2	—	4.5	—	4.7
Sterile controls.....	—	—	—	—	—	—	—	—

\* The numbers 1 and 2 refer to the glucose-free and the glucose-containing cultures, respectively.

TABLE 21  
FORMATION OF CREATIN IN PEPTONE CULTURES OF MICRO-ORGANISMS

Culture	2 Days Old		5 Days Old		8 Days Old		14 Days Old	
	1*	2*	1	2	1	2	1	2
<i>B. proteus-vulgaris</i> .....	—	2.2	Trace	3.9	5.9	5.3	8.5	3.1
<i>B. pyocyaneus</i> .....	—	6.8	—	15.7	—	†	†	†
<i>B. typhosus</i> .....	—	3.0	—	6.4	—	5.5	—	3.9
<i>B. coli-communis</i> .....	—	7.2	—	4.8	—	5.3	—	—
<i>Sp. cholerae</i> .....	—	2.3	—	8.6	4.7	6.5	—	†
<i>B. subtilis</i> .....	—	6.7	—	7.7	7.2	.....	†	13.1
<i>B. paratyphosus</i> .....	—	2.4	—	5.3	—	6.0	—	4.6
<i>B. cloacae</i> .....	—	3.9	—	5.0	—	8.8	—	—
<i>B. faecalis-alkaligenes</i> .....	—	9.8	—	—	—	—	—	—
<i>Sp. metchnikovii</i> .....	—	10.1	—	4.3	—	0.4	—	7.5
<i>Staph. pyogenes</i> .....	—	7.6	—	1.5	—	—	—	1.8
<i>B. dysenteriae, Shiga</i> .....	—	7.3	—	1.9	—	0.3	—	5.8
<i>B. acidi-lactici</i> .....	—	5.4	—	2.8	—	—	—	3.2
<i>B. prodigiosus</i> .....	—	10.7	—	2.1	—	—	—	7.1
Sterile controls.....	—	—	—	—	—	—	—	—

\* The numbers 1 and 2 refer to the glucose-free and the glucose-containing cultures, respectively.

† When the reagent was added to these samples, a very dark color was produced, which could not be matched with the standard creatinin solution.

There seem to be two possible explanations of this surprising effect of glucose on creatin- and creatinin-production by micro-organisms. Either these substances are regularly produced by bacteria in peptone solutions and their decomposition as fast as formed prevented by the presence of sugar, or else glucose, or some of its split products, actually takes part in the synthesis by the organisms of these two nitrogenous

compounds. The former seems to be the more probable of the two hypotheses. Fortunately, this proposition is susceptible of proof; at least, strong evidence for or against it may be obtained by investigating the effect of glucose on the decomposition by micro-organisms of quantities of creatin and creatinin added to peptone solution.

TABLE 22  
THE DECOMPOSITION OF CREATININ BY BACTERIA

Culture	2 Days Old		6 Days Old		15 Days Old	
	Glucose Present	Glucose Absent	Glucose Present	Glucose Absent	Glucose Present	Glucose Absent
<i>B. proteus-vulgaris</i> .....	52.4	47.1	36.9	31.9	32.3	32.3
<i>B. pyocyaneus</i> .....	51.3	53.3	29.3	38.3	*	Trace
<i>B. typhosus</i> .....	48.1	53.9	42.0	42.4	48.2	50.0
<i>B. coli-communis</i> .....	41.3	56.2	33.6	44.6	32.9	Trace
<i>B. subtilis</i> .....	41.8	54.2	51.8	53.6	54.8	57.1
<i>B. faecalis-alkaligenes</i> .....	56.5	54.1	46.8	55.5	48.8	52.2
<i>Staph. pyogenes-aureus</i> .....	47.4	13.4	48.7	Trace	44.1	Trace
<i>B. dysenteriae, Shiga</i> .....	56.9	53.6	48.1	51.4	51.8	51.6
<i>B. acidilactici</i> .....	50.7	45.2	53.2	32.7	58.2	34.8
Sterile medium.....	54.6	54.6	55.6	55.5	55.1	55.3

\* The solution was too dark-colored to be comparable with the standard.

An experiment similar to the one just described, but with a 2% peptone solution containing in each liter the juice from 1 lb. of lean beef as the medium used, was carried out. Only creatinin determinations were made. The same method was used as in the former experiment. Samples of 2 c.c. were used for most of the determinations.

Table 22 gives the results of the test. As in all the preceding tables, the values are given in milligrams per 100 c.c. of the culture fluid. The data seem to be in accord with the explanation already suggested of the effect of glucose on the formation of creatin and creatinin by bacteria. The latter compound is decomposed in most cases to a considerably less extent when glucose is present in the medium. The very great effect of this substance in the case of the staphylococcus cultures is worthy of special mention. It is also interesting that the cultures of *B. faecalis-alkaligenes* and of *B. dysenteriae* show the same absence of the sparing effect of sugar as they did in the case of the peptone solutions.

Further experiments are now in progress on this phase of bacterial metabolism, and it is to be hoped that more definite information may be obtained on the part played by glucose in these reactions.

#### SUMMARY

Peptone cultures of most bacteria give fluctuating concentrations of amino-acid, showing that these bodies are formed and broken down continuously by the organisms.

A few strongly proteolytic organisms are exceptions to the rule in that their cultures show steadily increasing concentrations of amino-acid. Among these are *B. pyocyaneus*, *B. subtilis*, *Sp. cholerae*, and *Sp. metchnikovii*.

Most species, when grown in peptone or peptone gelatin media, show an inclination to utilize the simpler compounds of nitrogen before attacking the protein or peptone.

Most species also show evidences of a continuous utilization of ammonium salts in small amounts.

The general phenomenon of the protein-sparing effect of glucose is evident in most cultures not only from their concentrations of free-ammonia, but also from their concentrations of amino-acid, and, in fact, may be disclosed by the latter when the former fails to give evidence of it, as is true in the case of *B. faecalis-alkaligenes* and *B. dysenteriae*, Shiga.

Practically the same characteristics of ammonia- and amino-acid-production are shown by the organisms on peptone solutions containing 5% gelatin as on pure peptone solutions, except that the concentrations in the former media are much greater in the case of those organisms having a gelatin-liquefying power.

The ammonia and amino-acid curves of *B. pyocyaneus* grown aerobically do not differ materially from those of the same organism grown anaerobically.

*B. welchii*, when grown under favorable conditions, shows very strong proteolytic activity.

The free ammonia and amino-acid curves of most micro-organisms give evidence of the existence in their cultures of large amounts of nitrogenous products intermediary between amino-acid and ammonia.

Urea and uric acid are not found in peptone cultures of bacteria, probably because of the ease with which these substances are decomposed by most species.

No method could be found for the detection and determination of allantoin which was applicable to peptone cultures or to cultures containing asparagin.

A few species of bacteria are capable of producing creatin and creatinin in sugar-free peptone cultures. Many more are capable of producing these substances in peptone media containing glucose, the probable reason for this effect of the sugar being its sparing action for the two compounds in question.

# MYXOMA-LIKE GROWTHS IN THE HEART, DUE TO LOCALIZATIONS OF SPIROCHAETA PALLIDA \*

PLATES 1 TO 4

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In 5 cases of congenital syphilis of infants round translucent nodules have been found in the myocardium, appearing to the naked eye as myxoma-like growths. Three cases were new-born infants dying a few days after birth, one was a child 14 months old, and one a child 18 months old. Of these cases 4 presented signs of syphilis and showed the presence of spirochetes in other organs and tissues; while 1 showed syphilis only in the myocardium.

The myxoma-like nodules occurred in all cases in the anterior wall of the left ventricle, either near the apex or near the ventricular septum. In 1 case a similar nodule was found on the posterior wall of the left ventricle. In 3 cases there were two distinct nodules in the anterior wall of the left ventricle, one just above the apex, the other higher, over the septum. The nodules, which appeared to be nearly round or ovoidal, spherical on section, were all elevated above the general level of the epicardium. The largest were 1 cm. in diameter; the smallest was 5 mm. They extended entirely through the heart-wall from epicardium to endocardium, projecting inwardly as well as outwardly on the epicardial surface. They were light-grayish in color, of a translucent and jelly-like quality, with a few prominent, tho small, blood-vessels running over or around them. Their consistence was that of mucoid tissue. In all respects they resembled small myxomas, and the first ones seen were at first sight so regarded. The hearts of the five infants were much enlarged, dilated, and hypertrophic, the walls of the left ventricle being 2 or 3 times thicker than normal. Smaller translucent areas also occurred throughout the myocardium.

On microscopic examination these round translucent areas were found to consist of an edematous gelatinous connective tissue, showing remains of atrophic muscle fibers, especially at the periphery. Microscopically the nodules were distinctly intramural, not quite reaching the

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endocardium, being separated from it by a thin band of heart muscle. A similar intervening thinner band was also present on the epicardial side. In the center of some nodules the muscle had completely disappeared. The remaining muscle fibers were arrayed somewhat like the meridians of longitude on a globe, farther apart at the equator, and converging at the poles. The space between the pressed-apart fibers was filled in with the mucoid connective tissue. Mucin stains, particularly cresyl violet, gave a strong mucin reaction. The gelatinous tissue was not very cellular, except in areas, the cells being chiefly varied forms of fibroblasts (epithelioid cells), lymphocytes, and plasma cells. Many of the fibroblasts had numerous branching processes, but the branching was not so marked as that in ordinary gelatinous tissue. The intercellular substance was semifluid or mucoid, or consisted of delicate fibrillae in a semifluid matrix. The histologic changes were precisely the same in kind as those between the heart-muscle fibers in spirochetosis of the myocardium, tho differing in degree, in greater mucin content, sharper localization, and greater destruction of muscle fibers. The changes in the muscle fibers indicated a process that was spreading peripherally from a center of more marked change. This central, presumably older, area showed no signs of caseation, fatty change, or fibrosis; it appeared simply more semifluid, or mucoid, than the peripheral portions. No new formation of blood vessels was shown. The cellular infiltration and proliferation were so slight that microscopically the nodule might easily have been taken for an edematous or hydropic area, or, when mucin stains were used, for a myxomatous area in the myocardium.

In sections prepared according to Levaditi's method, these mucoid areas were found to be localized colonies of *Spirochaeta pallida* occurring in enormous numbers. The smaller mucoid patches throughout the myocardium likewise showed *Spirochaeta pallida* in great numbers. The larger round sharply localized mucoid areas might be regarded as representing a mucoid granuloma or myxogumma, altho there was no histologic resemblance to the ordinary gumma of late syphilis.

In the literature I have been unable to find any microscopic description exactly fitting these lesions. In the older literature on syphilis, there are not infrequent macroscopic descriptions of the gummata of late acquired syphilis as being semitranslucent, gelatinous, or mucoid in character, but the microscopic picture of such macroscopic gelatinous gummata as described does not in any way resemble these lesions.

Only one instance occurs in the literature of an observation of a syphilitic lesion that was most probably of the same character as those found in my cases.

Shattuck<sup>1</sup> exhibited before the Pathological Society of London, in 1881, a specimen of a "mucous tumor of heart (syphilitic gumma?)," with the following description:

"The heart of an infant, in connection with the right ventricle of which there had been formed a small circumscribed, slightly nodular and granulated ovoidal tumor, about a centimeter in its chief diameter, which slightly overhangs its base and projects into the ventricle from the anterior wall immediately below the corresponding cusp of the pulmonary valve, the inferior side of which it also involves.

"*Histology*.—A net-work of delicate connective-tissue in the meshes of which lie large many-branching, granular, indented cells, of which the processes form a secondary, closer reticulum. The other organs were healthy. From a child congenitally syphilitic."

Shattuck's observation was made long before the spirochete became the positive identification of a syphilitic lesion, yet his gross and histologic descriptions make it extremely likely that the lesion he described is of the same nature as the mucoid nodules in my five cases, in which *Spirochaeta pallida* occurred in great numbers, proving conclusively that the lesions were due to syphilis.

If myxoma-like nodules can be produced in the heart-wall by local colonization of spirochetes, the question at once arises as to the true nature of the mucoid nodules described under the heading of myxomas of the heart-wall. The myxoma is regarded as one of the common forms of the relatively very rare primary neoplasms of the heart; but the real nature of these neoplasms has occasioned much discussion among pathologists. I have found reports of 48 such cardiac myxomas in the literature. Of these, 24 were recorded found in the left auricle, 3 in the left ventricle, 2 in the right auricle, 2 in the right ventricle, 1 involving both auricles, 3 on the aortic valves, 2 on the mitral, 8 on the tricuspid, and 3 on the pulmonary valves. The majority were endocardial in origin, either firmly connected with the endocardium, or rising therefrom by a pedicle, giving the mucoid nodule the appearance of a polypoid myxoma. Only in a few cases has the apparent myxoma been imbedded in the heart-wall or shown such intimate relationship as to convey the impression that the growth was intramural, not endocardial, in origin.

These so-called myxomas of the heart have been small in size, and clinically unrecognizable, being discovered only by accident at autopsy. They have been called myxomas because of their translucency and their histologic appearance of branching cells, with fine fibrillae and semifluid intercellular substance. Staining tests for mucin have either

<sup>1</sup> Tr. Path. Soc. London, 1881, 32, p. 77.

not been tried, or have resulted negatively, with the exception of Hanser's<sup>2</sup> case in which positive mucin tests with mucicarmin and thionin were obtained. These reactions Hanser used as positive argument to prove the true myxoma nature of the little polypoid translucent nodule, the size of a cherry stone, which he had found springing from the anterior papillary muscle of the mitral. The location of the growth, the mucin reaction, the absence of blood pigment, and the presence of elastic fibers he regarded as proof of the true neoplastic character of this tumor.

On the other hand, it is asserted that the majority of these endocardial mucoid nodules represent organized thrombi, or inflammatory proliferations of the endocardium, those on the valves in part belonging to the so-called "Lambl's excrescences." I myself have had 2 cases of endocardial mucoid nodules undoubtedly originating as organizing thrombi.

Case 1.—Old acquired syphilis. Angina pectoris. Sudden death. Chronic syphilitic myocarditis. The right auricular appendix was completely filled with a translucent mucoid tissue, attached to the surface of which the remains of an old thrombus could still be seen with the naked eye. Microscopically, the mucoid area presented the appearance of myxomatous tissue, giving good reactions for mucin, mostly devoid of any blood pigment or fibrin, and containing new-formed elastic fibers. Between the myxomatous connective tissue and the thrombus lay an intermediate area of beginning organization and formation of a young gelatinous fibroblastic tissue. Here there could be not the slightest doubt that the myxomatous tissue represented simply the organization of a thrombus attached to the endocardium, for all stages of the process of the organization and development of the mucoid tissue could be seen. As this observation took place before the discovery of the spirochete no examination for spirochetes was made.

Case 2.—Old acquired syphilis. Angina pectoris. Sudden death. Gummatous myocarditis of left ventricle. Thrombosis of left ventricle. Organizing thrombus attached to thinned heart wall just above apex. Next to the heart wall, which at this point was mostly fibroid, the organizing thrombus presented a layer of mucoid tissue, giving reactions for mucin, in chief part containing no blood pigment, and showing new-formed elastic fibrillae. No examination for spirochetes was made.

There can be no doubt that endocardial nodules of mucoid tissue arise from the organization of parietal thrombi, and it is most probable that all the reported cases of cardiac myxoma in which the supposed neoplasm was attached to the endocardium, were not neoplasms, but polypoid formations of a young mucoid fibroblastic tissue, arising from the organization of a thrombus.

<sup>2</sup> Frankfurter Ztschr. f. Path., 1911-12, 9, p. 362.

Fibroblastic proliferations of the endocardium, particularly of the valvular endocardium, show a particular tendency toward the formation of a mucoid connective tissue that gives a more or less pronounced staining reaction for mucin. It has been my experience that in every organizing valvular vegetation, some of the new-formed connective tissue gives in its earlier stages such mucin-staining reactions. The occurrence of mucin in organizing cardiac thrombi has been noted by other writers. Wegelin<sup>3</sup> states that "the mucin-reactions often give a positive result in the organization of thrombi of the heart wall." Brenner, Czapek, Curtis, Lubarsch, and others, also recognize the mucoid character of organizing thrombi.

In not a single one of the reported cases of supposed myxoma attached to the endocardium is the blastoma nature of the mucoid tissue conclusively demonstrated. On the contrary, the evidence given points to the origin from organizing thrombi. They should therefore be rejected as true cardiac neoplasms, and no longer classed with such. A true myxoblastoma of the endocardium is yet to be demonstrated. This applies also to the relatively few intramural mucoid nodules reported as myxomas. From the given descriptions I do not believe that any of them has been a true blastoma. My own five cases show conclusively that mucoid nodules, giving positive mucin tests, occur in the myocardium in cases of congenital syphilis and represent formations of a mucoid fibroblastic tissue due to the localization of large colonies of *Spirochaeta pallida*. The few intramyocardial myxomas reported (Czapek, Hlava) were probably of the same nature as the nodules which I have described, but Shattuck's case is the only one in which a syphilitic origin was suspected, and the only one in which we may be reasonably sure that the pathologic conditions were the same as in my cases.

I have shown elsewhere that in congenital myocardial syphilis the new-formed interstitial connective tissue is frequently mucoid or edematous in type, and gives a mucin reaction with cresyl violet. In the average case these mucoid proliferations are too small to be seen individually with the naked eye, but may be so extensive as to make the entire heart wall more translucent and lighter in color, as shown in Fig. 2. Larger, more sharply localized colonies of spirochetes rarely produce the larger localized mucoid nodule seen in my cases. These myxogummas are probably relatively rare, or they could hardly have escaped observation to the degree that they have; yet I believe that

<sup>3</sup> Frankfurter Ztschr. f. Path., 1911-12, 9, p. 117.

more detailed examinations of the hearts of congenitally syphilitic infants and children will show a more common occurrence than we now suppose. In my own experience, the myxogumma of congenital syphilis is more frequent than the caseating gumma of the heart in acquired syphilis, of which I have seen only one example.

#### SUMMARY

Localized colonization of *Spirochaeta pallida* in the myocardium in congenital syphilis may lead to the formation of an intramural mucoid nodule, from 5 to 10 mm. in diameter, resembling mucoid tissue both macroscopically and microscopically, and giving a positive mucin reaction. Such myxogummas are relatively rare, and may be mistaken for myxomas.

A mucoid fibroblastic tissue giving positive mucin reactions is very commonly produced in the organization of parietal cardiac thrombi and valvular vegetations. The absence of blood pigment, the positive mucin reaction, and the presence of newly formed elastic fibers cannot, however, be taken as points deciding the blastoma character of the mucoid tissue.

There is no positive proof of the occurrence of a true myxoblastoma of the heart. The mucoid nodules attached to the endocardium are undoubtedly organized thrombi; the rarer ones in the myocardium may most plausibly be interpreted as myxogummas, or proliferations of a mucoid fibroblastic tissue caused by the localization therein of *Spirochaeta pallida*.

## EXPLANATION OF PLATES

## PLATE 1

FIG. 1. Heart of infant dying from congenital syphilis. Two mucoid nodules (myxogummas) in anterior wall of left ventricle near septum. Left ventricle hypertrophic and dilated.

FIG. 2. Heart of infant dying from congenital syphilis. Two mucoid nodules in anterior wall of left ventricle, above apex and involving part of septum. Entire heart light-colored and more translucent than normal as a result of diffuse interstitial mucoid proliferation or edema caused by diffuse spirochetosis. Mucoid nodules contained enormous numbers of spirochetes.

## PLATE 2

FIG. 3. Transverse section of mucoid nodule seen in Fig. 1. Nodule extends through myocardium, is elevated on epicardial and endocardial sides, but separated from epicardium and endocardium by narrow bands of muscle. The bluish fibrillar areas in the nodule are the mucoid areas containing spirochetes. Bands of pale atrophic muscle found throughout entire translucent area. The appearances are those of localized myxedema of the myocardium with atrophy and degeneration of muscle rather than of localized granulomatous proliferation (gumma). Hematoxylin and eosin.

## PLATE 3

FIG. 4. Section of translucent area stained with cresyl violet. Pink fibrillae show marked mucin reaction between muscle fibers.

## PLATE 4

FIG. 5. Section of the myxoma-like nodule in Fig. 2, from half of nodule prepared by Levaditi's method. Drawing made from photomicrograph showing actual number of spirochetes appearing in one focal field, with those partly out of focus represented as in one focal plane.

PLATE I



Figure 1

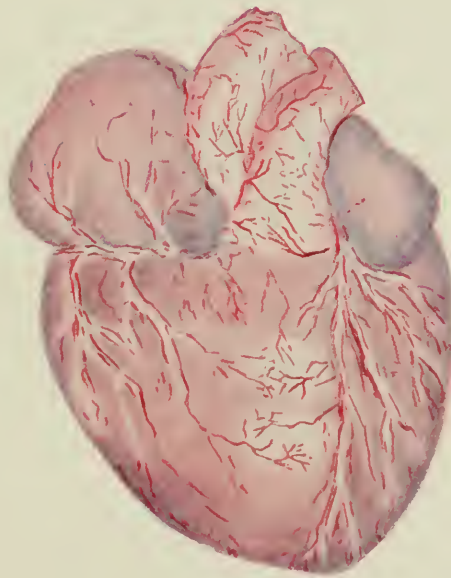


Figure 2



PLATE 2

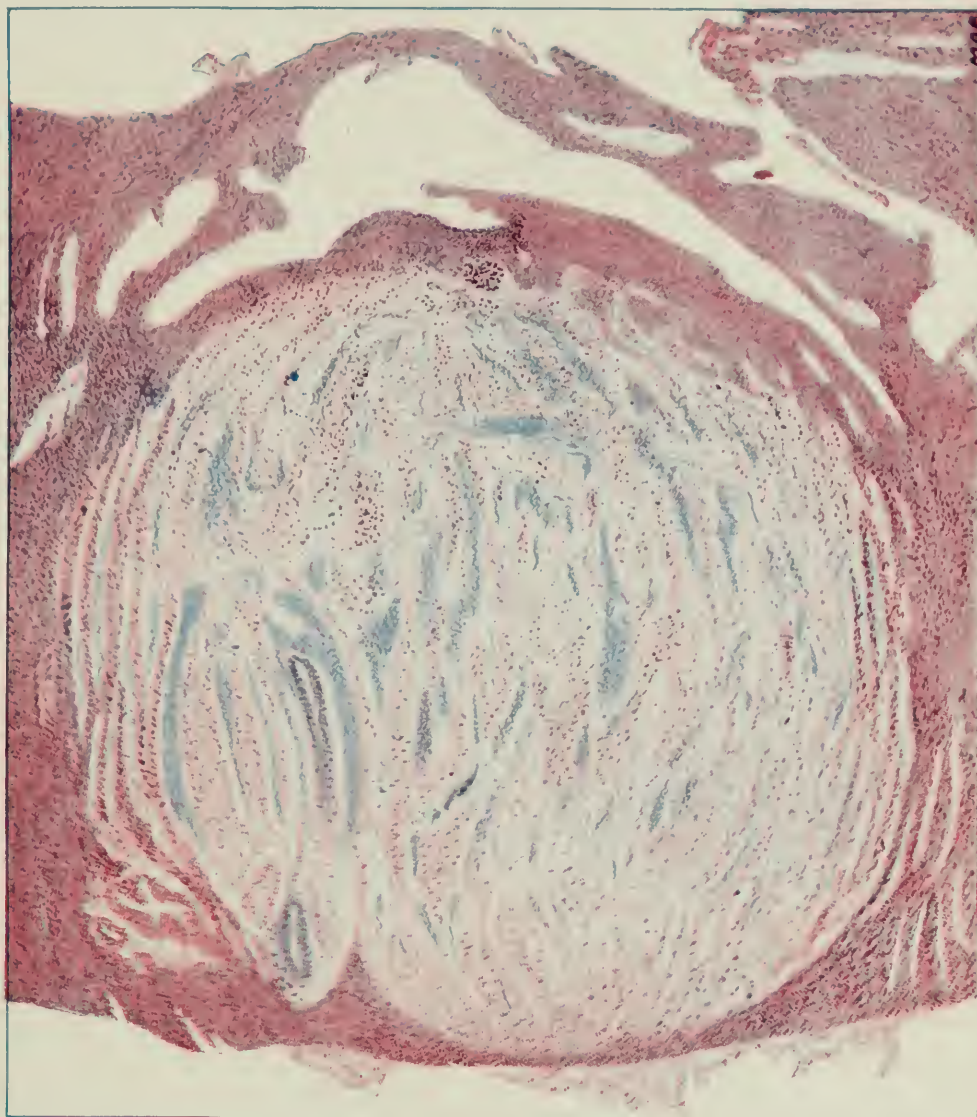


Figure 3



PLATE 3

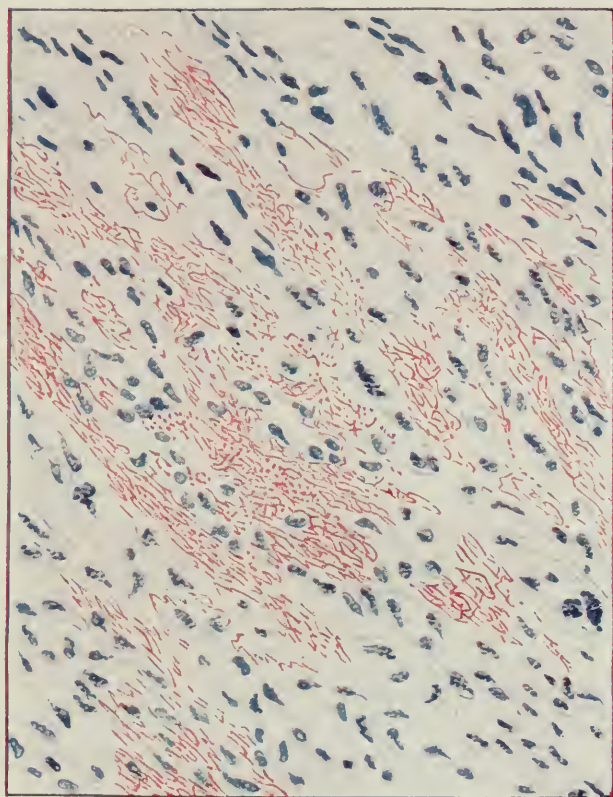


Figure 4



PLATE 4

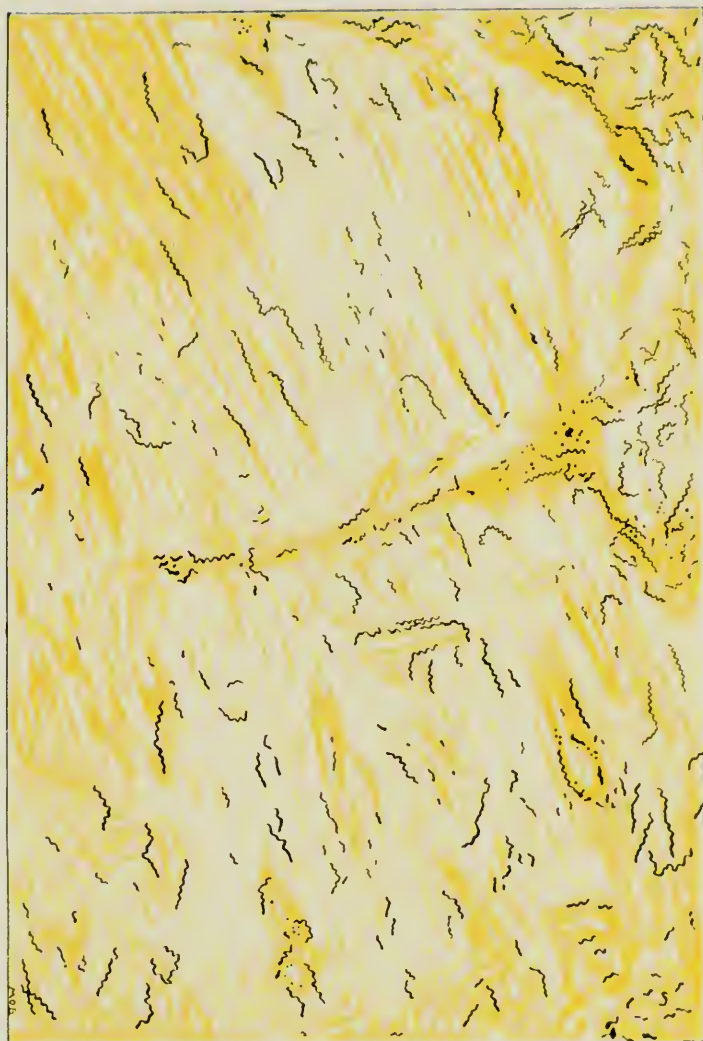


Figure 5



## FURTHER STUDY ON EXPERIMENTAL CHOLERA-CARRIERS \*

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The early investigations concerning cholera infection in animals were undertaken with a view to establishing the etiologic relation between Koch's comma bacillus and the disease known as Asiatic cholera.

The feeding experiments of Thiersch<sup>1</sup> on mice, Richards on hogs,<sup>1</sup> Wiener<sup>2</sup> on cats, and Karlinski<sup>3</sup> on dogs have not been successfully repeated. Nikati and Rietsch<sup>4</sup> asserted that by injection of cholera vibrios into the intestine of guinea-pigs they had produced a disease resembling Asiatic cholera in man. R. Koch<sup>1</sup> fed cholera cultures to guinea-pigs after having introduced a considerable amount of sodium bicarbonate into the stomach to eliminate the action of its acid. The animals also received opium intraperitoneally. According to Koch, they died of cholera infection. Thomas<sup>1</sup> found that intravenous injection of small amounts of cholera culture killed rabbits; cholera vibrios were found in the intestine, and the animals exhibited pathologic lesions in the intestine which resembled those in fatal human cases. Issaeff and Kolle<sup>5</sup> confirmed these findings, being particularly successful when using young rabbits. Metschnikoff,<sup>1</sup> who infected suckling rabbits by feeding cholera cultures, stated that healthy rabbits brought into close contact with the infected animals died of cholera infection.

The experiments of Violle<sup>6</sup> have a decided bearing on the present question. Violle studied the immunity which results from inoculation of cholera vibrios and other bacteria into the gall-bladder with ligated cystic duct. Vibrios were found in the ligated gall-bladder up to the 15th day after inoculation. The resulting immunity was considerable.

In a previous communication the results of experiments were published which showed the relative values of various methods tried in our endeavor to make cholera-carriers of guinea-pigs. The duration of the state of parasitism following intravesicular inoculation, the distribution of cholera vibrios throughout the alimentary canal, and the elimination of cholera vibrios in the feces of the experimental cholera-

\* Received for publication February 5, 1916.

<sup>1</sup> Cited from Kolle-Schürmann, Kolle and Wassermann's Handb. d. pathogen. Mikroorganismen, 1912, 4, p. 1.

<sup>2</sup> Centralbl. f. Bakteriöl., 1896, 19, pp. 205, 595.

<sup>3</sup> Ibid., 1896, 20, p. 150.

<sup>4</sup> Deutsch. med. Wchnschr., 1884, 10, p. 634. Semaine méd., 1884, 4, p. 393.

<sup>5</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1894, 18, p. 17.

<sup>6</sup> Ann. de l'Inst. Pasteur, 1912, 26, pp. 381, 467.

carriers were studied. Further investigations bearing on the nature of this condition are recorded here. In the course of our experiments it became desirable to perform repeated intravenous injections, a procedure hardly practicable with guinea-pigs. Rabbits were substituted, with a consequent necessity of having to repeat on rabbits some of the experiments already published, in order to compare the findings with those obtained in the case of guinea-pigs.

#### TECHNIC

The technic used here was essentially the same as that used previously. The intravesicular inoculation alone was employed. Blood was obtained by bleeding the guinea-pigs from the carotid artery. The peritoneal cavity was examined for presence or absence of cholera vibrios in the following way: The cavity was opened with strictly aseptic precautions and washed out with 1 c.c. of peptone water, which was then planted into a tube containing peptone water. About half of the spleen was crushed by means of a sterile forceps, and a piece of the left lobe of the liver was cut into small pieces; both were then planted in peptone water. The pleural cavity was opened separately, and a part of the left lower lobe of the lung was planted in the same way as the spleen and the liver. Thus the cultures from peritoneum, liver, lung, and spleen, and the blood cultures, were made before the alimentary canal proper was opened.

#### EXPERIMENTS ON GUINEA-PIGS

In our first experiments the animals remained apparently in good health after the intravesicular inoculation. At autopsy the guinea-pigs showed no particular changes of the internal organs which could not be explained as due to the surgical encheiresis. The gall-bladder, which was of normal size, contained bile of normal appearance, except in a few instances in which the bile was flaked with white.

In the course of further experiments a culture freshly isolated from an animal's gall-bladder was used for the inoculation of the next series of animals. Thus an intermittent passage through guinea-pigs' gall-bladders and ox-bile culture medium was carried on. The results of inoculation into the gall-bladder of the same strain of cholera vibrio after several passages through animals' gall-bladders differed from those in the earlier experiments. Most of the guinea-pigs which were inoculated with a culture previously passed through several animals were decidedly ill after the inoculation, some dying in from 3 to 4 days after inoculation.

There was a more or less extensive swelling over the abdomen, due to hemorrhagic edema. Numerous cholera vibrios were found in smears and cultures from the edema. The peritoneum was congested and fibrinous exudate covered the surface of the liver and the omentum. The internal organs were congested, the large soft spleen dark-red in color. Microscopic examination of the exudate

revealed comparatively few, mostly phagocytosed, vibrios. Cholera vibrios were recovered by culture from the internal organs, peritoneum, blood, and subcutaneous edema. In these cases the infection of the peritoneum and the subcutaneous tissue, injured by operation, was due no doubt to leakage of a small quantity of the cholera culture during the inoculation. Most of the animals survived, and autopsy performed on them later revealed far more extensive changes of the gall-bladder than those noticed in our earlier experiments. The gall-bladder was rather small, and its peritoneal cover showed considerable congestion. It contained a small amount of thick bile of light-yellow color, which under the microscope was found to consist of amorphous debris, desquamated epithelial cells, and leukocytes in various stages of degeneration. As a rule, typical vibrios, isolated or in clusters, were seen in the smears.

The liver in some cases exhibited pronounced gross pathologic lesions. Small yellowish foci resembling miliary abscesses were scattered throughout the liver tissue, forming confluent areas in some instances. The spleen was slightly enlarged, but firm. Smears made from the diseased parts of the liver revealed the presence of typical vibrios.

*Examination of Microscopic Sections.*—The gall-bladder exhibited various degrees of inflammation. In some cases the epithelium was well preserved, the mucous membrane showing only a slight degree of cellular infiltration. In other cases the epithelium was fairly preserved, but the surface of the mucous membrane was covered with numerous pus cells. The tissue proper of the mucosa showed a high degree of cellular infiltration and hemorrhage. In other sections the epithelium was missing, and the polymorphonuclears predominated among the cellular elements of the mucosa. Strips of cellular infiltration penetrated into the submucosa.

The liver also showed various degrees of pathologic changes. Small aggregates of leukocytes were scattered throughout the section, located chiefly on the periphery of the acini and at times suggesting direct connection with the small branches of the bile ducts. Occasional comparatively large necrotic areas were encountered. They had a distinct hemorrhagic halo and were surrounded by a zone of cellular infiltration in which the polymorphonuclear leukocytes were predominant. Large deeply stained bodies were sometimes encountered in the peripheral zone of these foci, of a questioned parasitic nature.

It was impossible to decide in how great a degree the changes described were due to infection by cholera vibrios. Some of the lesions in the liver were of too chronic a character to be primarily caused by the cholera vibrios in so short a time. Nevertheless, the presence of cholera vibrios in the smears and cultures from the diseased parts of the liver indicates that these organisms played a part in producing the lesions. They may have found a locus minoris resistentiae in the pre-existing lesions. Except in animals dying of general infection shortly after inoculation, no cholera vibrios were found in the blood, peritoneum, spleen, or lung. In the liver they were found occasionally.

An attempt was made to estimate the number of the cholera vibrios in the various sections of the alimentary canal.

Smears from the gall-bladder, duodenum, and ileum, were examined under the microscope. Equal and constant amounts of the contents of the gall-bladder, duodenum, ileum, and cecum were smeared directly on Dieudonné and plain alkaline-agar plates. The Dieudonné plates were intended to show the number of cholera colonies exclusive of other intestinal bacteria; the plain agar plates, the number of bacteria other than cholera vibrios in the digestive system of the animals; and the microscopic examination of the smears was to demonstrate the presence of any bacteria which might refuse to grow on the plates, whether on account of the alkaline reaction of the media or aerobic methods of cultivation.

The vibrios were almost constantly present, occurring in greatest numbers in the gall-bladder and the ileum. The duodenum and the cecum sometimes contained numerous cholera vibrios; at other times none. The number found in the various parts of the alimentary canal varied greatly with individual animals.

The following tables show also that cholera vibrios are practically the only bacteria present in the proximal part of the alimentary canal of experimental cholera-carriers during life. Smears and plate cultures directly from the bile, and from the contents of the duodenum and the ileum showed cholera vibrios in pure culture in every case in which autopsy and bacteriologic examination were made immediately after the death of the animal. In our first paper we stated that the large intestine, including the cecum, harbored bacteria grossly resembling vibrios; microscopic examination of the contents of the cecum was therefore disregarded. These micro-organisms and fusiform bacilli were encountered frequently, as well as micrococci and rod-shaped bacteria in the smears and peptone cultures, but not on the Dieudonné agar plates. Plain agar plates inoculated directly from the cecum showed at times remarkably few colonies other than cholera vibrios.

Under normal conditions, then, the cholera vibrios greatly predominate in the proximal part of the alimentary canal; the competition with other intestinal bacteria begins to take place in the large intestine. This factor, as well as the consistency of the contents in the large intestine, is responsible for the irregularity with which the cholera vibrios appeared in the feces of the experimental cholera-carriers.

The number of cholera vibrios in the gall-bladder and in the intestine, as before stated, varied considerably with individual animals. In some cases the cholera vibrios were evidently merely passed from the gall-bladder into the intestine, while in other instances they were present in the intestine in great numbers as tho they had multiplied in the

intestine. Among the numerous guinea-pigs examined not a single animal was encountered in which cholera vibrios were found only in the gall-bladder and not in the intestine; in other words, there was no evidence that the inflammatory process in the gall-bladder had arrested the function of the organ. All guinea-pigs examined within 14 days after inoculation harbored cholera vibrios in the gall-bladder and in the intestine. Those guinea-pigs which were examined later than 14 days after inoculation, and were found negative, showed eventual signs of inflammation of the gall-bladder, but the organ was maintaining its function; that is, free communication evidently existed between the gall-bladder and the intestine.

#### EXPERIMENTS ON RABBITS

The following experiments cover the duration of the carrier state and the nature of the condition in rabbits.

#### TECHNIC

The intravesicular inoculation is technically more difficult in rabbits than in guinea-pigs because the anatomic conditions make it necessary to open the peritoneal cavity by a large incision, and to perform the injection proper within the peritoneal cavity. The gall-bladder in rabbits is oval in shape, firmly adherent to the liver; the cervix is buried in the liver tissue. The cystic duct branches off on the side of the distal end of the gall-bladder. Thus the cervix has the shape of a diverticulum. The guinea-pig's gall-bladder is pear-shaped, narrowing gradually towards the cystic duct. These anatomic differences are probably responsible for the difference in behavior between guinea-pigs and rabbits toward the intravesicular inoculation of cholera vibrios.

The inflammatory process of the gall-bladder which follows the intravesicular injection of cholera vibrios was of much greater intensity in rabbits than in guinea-pigs, bringing about the occlusion of the gall-bladder in the majority of the animals. The communication between the gall-bladder and the intestine was interrupted, so that the intestine was free from cholera vibrios at a time when they were still present in the contents of the gall-bladder.

The gall bladder at this period was usually smaller than normal, and its wall, including the mucosa, thickened. It contained a thin colorless liquid and a creamy sediment, which consisted of pus cells in various stages of degeneration, and of amorphous debris. At times the gall-bladder was filled with thick inspissated pus, composed of pus cells, fibrin, and amorphous debris. In this stage the gall-bladder represents a focus, completely encapsulated by the thickened wall, in which the cholera vibrios remain alive for a certain time, but are no longer passed into the intestine.

TABLE 1  
RESULTS OF EXAMINATION OF VARIOUS ORGANS OF EXPERIMENTAL CHOLERA-CARRIERS FOR THE PRESENCE OF CHOLERA VIBRIOS

Guinea-pig	Days After Inoculation	Culture*	Gall-bladder	Duodenum	Ileum	Cecum
69	3	63	+	+	+	+
68	4	63	+	+	+	+
65	5	57	+	+	+	+
78	6	4	+	+	+	+
67	7	63	+	+	+	+
70	8	63	+	+	+	+
66	...	57	+	+	+	+

\* In this and the following tables the number of the culture corresponds with the number of the last guinea-pig from whose gall-bladder the culture was recovered.

KEY TO TABLES 1, 2, AND 3  
— = cholera vibrios not found.  
+ = cholera vibrios present.

TABLE 2  
ESTIMATION OF THE NUMBER OF CHOLERA VIBRIOS IN THE VARIOUS SECTIONS OF THE ALIMENTARY CANAL

Guinea-pig	Days After Inoculation	Culture	Dieudonné Plates			
			Gall-bladder	Duodenum	Ileum	Cecum
108	1	4	0	0	0	0
104	2	4	0	0	0	0
111	2	4	0	0	0	0
96	4	57	Num.	—	Num.	Few
97	4	57	Very num.	Num.	Few	Very few
94	4	57	Num.	Few	Very num.	Num.
119	4	4	Very num.	Num.	Very num.	Few
106	6	4	Num.	—	Num.	Very few
107	6	4	0	0	0	0
113	6	4	0	0	0	0
80	7	4	Very num.	Very few	Very num.	Num.
88	7	57	Very num.	Few	Few	Very few
115	7	4	0	0	0	0
112	7	4	Very num.	Very num.	Very num.	Very num.
99	8	57	Very num.	Few	Few	—
100	8	57	Very num.	Num.	Very num.	—
98	9	57	Very num.	Num.	Num.	—

TABLE 3  
THE DISTRIBUTION OF CHOLERA VIBRIOS THROUGHOUT THE ORGANS OF INOCULATED RABBITS AND THE DURATION OF THE STATE OF CARRIER

Rabbit	Days After Inoculation	Culture	Direct Plates			
			Gall-bladder	Duodenum	Ileum	Cecum
9	3	4	Num.	Num.	Num.	Num.
14	5	4	Num.	Very few	Few	Very few
11	9	4	—	—	—	—
13	10	4	Few	—	—	—
2	13	4	0	0	0	0
10	13	4	Num.	—	—	—
12	14	4	—	—	—	—
3	57	4	—	—	—	—



## CONCLUSION

Sufficient evidence has been gathered, it is believed, to justify the opinion that the condition in question is an infection of the gall-bladder, and that the cholera vibrios injected into and recovered by culture from the gall-bladder, stand in causal connection with the pathologic changes encountered, instead of merely surviving as saprophytes at the place of inoculation.

The infectious process may vary in extent and intensity; remain limited to the gall-bladder, or extend to the liver.

The absence of cholera vibrios from blood from the lungs, and from the spleen makes the septicemic character of the infection highly improbable.

With regard to the intravesicular inoculation the rabbit's behavior differed from that of the guinea-pig inasmuch as the inflammatory process which followed the injection of cholera vibrios brought about the occlusion of the gall-bladder so that the cholera vibrios were no longer to be found in the intestine at a time when they were still present in the contents of the gall-bladder.

It is evidently a benign process which shows marked tendency to healing; i. e. the cholera vibrios disappear from the animal's body and the animal survives, altho a large percentage of the animals showed signs of chronic intoxication.

# BACTERIOLOGIC FINDINGS IN OZENA \*

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Inquiry into the bacteriology of ozena has engaged the attention of a number of workers during the past 25 years.

In the early eighties of the last century, investigations on the part of Fränkel, Löwenberg, Friedländer, Weichselbaum, Paulsen, and particularly the work of Abel<sup>1</sup>, furnished the interesting suggestion that ozena was due to a bacillus called by Abel "Bacillus mucosus Ozaenae." This organism was recognized by him to be identical with Löwenberg's bacillus,<sup>2</sup> and closely related to Friedländer's pneumobacillus. Abel found it present in 100 cases of ozena, while in 250 normal controls he failed to discover it once. Paulsen<sup>3</sup> isolated *B. mucosus ozaenae* in 51 cases; Strazza<sup>4</sup> in 25, Löwenberg<sup>2</sup> in 16; Marano<sup>5</sup> in 10; Thost<sup>6</sup> in 12 of 17; and Hajek<sup>7</sup> in 7 of 10.

In contrast with these findings there is a marked absence of Abel's bacillus in infections other than ozena. Abel and Paulsen, reporting their own study of several hundred examinations and taking into account all work published up to that time, estimated that *B. mucosus* occurred in only 1% of nasal cases and in about 3% of oral cases. Netter<sup>8</sup>, in an examination of the sputum of 165 healthy persons, found Abel's bacillus 3 times. Rosenthal<sup>9</sup> in 14 cases found it in 3, these being tuberculous. Krause<sup>10</sup> studying 30 cases of influenza discovered it once. Kowalski likewise obtained it once in 16 cases of influenza. Podbielski<sup>11</sup> in 50 oral examinations found it once. Besser<sup>12</sup> investigating the secretions of 81 nasal cases obtained it twice. Wright<sup>13</sup> reports it totally absent in 10 cases. Paulsen in his study of 27 normal, and 24 acute catarrhal conditions failed to obtain a single culture. When Nicolle and Herbert<sup>14</sup> conducted their investigation of 1,600 cases of angina, this bacillus was found in pure growth in the mouths of 6 patients.

\* Received for publication February 5, 1916.

<sup>1</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1896, 31, p. 89. Centralbl. f. Bakteriöl., 1893, 31, p. 161.

<sup>2</sup> Deutsch. med. Wchnschr., 1885, 11, p. 22.

<sup>3</sup> Centralbl. f. Bakteriöl., 1890, 8, p. 344.

<sup>4</sup> Primo Congresso della societa italiana die Laringologia, 1893.

<sup>5</sup> Centralbl. f. Bakteriöl., 1890, 8, p. 179.

<sup>6</sup> Deutsch. med. Wchnschr., 1886, 12, p. 161.

<sup>7</sup> Berl. klin. Wchnschr., 1888, 25, p. 659.

<sup>8</sup> Cited by Baumgarten's Jahresbericht, 1890, p. 81.

<sup>9</sup> Ein Beitrag zur Kenntniss der Bakterienflora der Mundhöhle. Dissertation, 1893.

<sup>10</sup> Virchow's Arch. f. path. Anat., 1881, 85, p. 325.

<sup>11</sup> Centralbl. f. Bakteriöl., 1891, 9, p. 617.

<sup>12</sup> Ibid., 1890, 7, p. 151.

<sup>13</sup> New York Med. Jour., 1889, 50, p. 92.

<sup>14</sup> Ann. de l'Inst. Pasteur, 1897, 11, p. 867.

Stein<sup>15</sup> in 51 typical cases of ozena, recognized Abel's bacillus in 44 cases. His 35 healthy controls yielded but 2 cultures. Surveying the entire field he reported the presence of bacterial groups as follows:

Bacillus mucosus-ozenae (in ozena 44).....	47
Staphylococcus albus and aureus.....	43
Diphtheroid bacilli (in ozena 25).....	37
Micrococci, unidentified types.....	29
Streptococci and pneumococci.....	11
B. pyocyaneus .....	3
Bacillus mucosus-ozenae (uncertain type).....	4
Spore-bearing bacilli .....	2
Perez bacillus .....	0

Cobb and Nagel<sup>16</sup> in an extensive study of 90 cases of ozena, with special care obtained *B. mucosus-ozenae* in every examination. Page<sup>17</sup> isolated Abel's bacillus in 2 cases and supplied fermentation tests for its identification.

Baurowitz<sup>18</sup> reported Abel's bacillus present in 12 patients with ozena. Seven of these showed pronounced symptoms and the fetor was marked. From 6 he obtained organisms capable of developing the characteristic ozena odor while in culture.

A little later, Perez<sup>19</sup> reported his bacteriologic findings in 63 cases, 22 of which presented typical symptoms of ozena. The organisms are arranged in the order of their numerical occurrence:

Staphylococcus albus and aureus.....	37
Pseudodiphtheria varieties .....	27
Löwenberg-Abel bacillus (in ozena 17).....	25
Perez coccobacillus (in ozena 8).....	8
Coli-group strains .....	7
Streptococci .....	5
Pneumococci .....	5
B. pyocyaneus .....	5

Perez emphasized the finding of a bacterial species which was capable of reproducing the ozena odor while in culture media. He demonstrated that this organism, named by him *Coccobacillus foetidus Ozaenae* was pathogenic for rabbits, while Abel's bacillus was not. Furthermore, its action was selective, showing affinity for the nasal mucosa, and symptoms developed which simulated those in man. Injected rabbits developed acute and sometimes even chronic conditions, and Perez was able to recover his bacillus from the animal's nasal discharges and to identify it. No such results were to be obtained with Abel's bacillus.

Hofer<sup>20</sup> in a careful investigation of 14 cases isolated Perez' bacillus in 57%, and Abel's bacillus in 86%. Hofer verified Perez' work with rabbits, concluding with him that his organism was the most important factor in ozena.

Recently a few reports have appeared in this country. Guggenheim<sup>21</sup> reviewed Hofer's work and noted the finding of Perez' bacillus in a few cases of ozena. His preliminary animal experiments were also in accord with Hofer's. Horn<sup>22</sup>

<sup>15</sup> Centralbl. f. Bakteriöl., 1900, 28, pp. 726, 769.

<sup>16</sup> Ann. Oto., Rhinol. and Laryngol., 1912, 21, p. 463.

<sup>17</sup> Jour. Med. Research, 1912, 26, p. 489.

<sup>18</sup> Centralbl. f. Bakteriöl., 1895, 18, p. 719.

<sup>19</sup> Ann. de l'Inst. Pasteur, 1899, 13, p. 937.

<sup>20</sup> Wien. klin. Wchnschr., 1913, 25, p. 1011.

<sup>21</sup> Interstate Med. Jour., 1915, 22, p. 2.

<sup>22</sup> Jour. Am. Med. Assn., 1915, 65, p. 788.

at a recent meeting of the American Medical Association read a paper on the etiology of ozena, in which he favored Perez' bacillus as being the most important etiologic factor.

All these investigations show that the most important bacterial organisms come under two bacteriologic groups; the Friedländer group, represented by Abel's bacillus, and the *Bacillus-suipestifer* group, represented by Perez' bacillus. In order to extend the present bacteriologic survey in this field, I have studied 50 well-authenticated cases of typical atrophic rhinitis, the majority of which presented ozena conditions.

Pure cultures of the important species were identified biochemically. Special attention was given to the strains obtained from the first few cases. When experience had determined the leading cultural or morphologic characteristics, a less extensive series of tests sufficed for identification purposes. Sero-biologic titrations were employed in the admittance of new strains to the established class, especially in the case of Perez' bacillus. Dr. W. R. Murry and Dr. W. P. Larson, of Minneapolis, by sending me one of Hofer's original cultures, enabled me to establish identification, and so directly correlate our investigations. Pathogenic action was determined with rabbits, these having been reported as promising a differential value.

#### TYPES OF BACTERIA AND THEIR RELATIVE FREQUENCY IN ATROPHIC RHINITIS

A tabulation of the results shows the relative frequency with which the members of the various bacterial groups appeared. The microscopic picture presents the best record within our reach and is therefore to be preferred, were it not also true that morphologic uniformity prevents identification. The cultural picture is less representative, unimportant species becoming prominent and valuable ones disappearing. It seemed desirable, therefore, to combine the findings obtained by the two methods of study.

The bacteriologic groups are arranged in the order of their frequency in the 50 cases.

Diphtheroids .....	43
Staphylococci, all varieties.....	37
<i>B. mucosus-capsulatus</i> .....	30
Perez' bacillus.....	22
Streptococci, all varieties.....	20
<i>B. proteus</i> , all varieties.....	20
Pneumococci .....	20
<i>B. pyocyaneus</i> .....	12
Spore-bearing types.....	10
<i>M. catarrhalis</i> .....	5
<i>B. coli</i> types.....	5
<i>B. influenzae</i> .....	4

In addition to these predominant forms, there appeared a variety of other organisms whose occurrence was limited, or difficult to verify culturally.

The diphtheroid group appeared most frequently, a fact in accord with the findings of the earlier workers. The special morphology of this group, together with the use of blood-serum media, explains its predominance.

The micrococci, second in order of frequency, demonstrate that conditions are favorable for their growth and action. Both Perez and Stein have placed this group of bacteria second in their tables.

Abel's bacillus appeared 30 times. The presence of this organism has been considered as typical of bacteriologic conditions in ozena, even by those unwilling to ascribe to it any etiologic significance. The cultures on the agar plates were often pure, while the microscopic picture of the same material revealed a variety of forms. Cobb and Nagel found this bacillus present in 100% of their cases.

Perez' bacillus, seen first by Horowitz, appeared 22 times in my series. It was identified by culture 15 times.

Perez reports this bacillus as a small, easily stained, gram-negative coccobacillus, showing extreme morphologic variations. It is nonmotile and forms shining transparent colonies. Gelatin is not liquefied or lactose fermented. Milk is not coagulated. Potato shows an abundant yellowish moist growth. It is pathogenic for guinea-pigs, mice, pigeons, and rabbits. All broth cultures give off a characteristic fetid odor, especially in the presence of albuminous media.

Specific agglutinins were easily produced. On the receipt of Hofer's culture, a vaccine was prepared and injected subcutaneously into rabbits. Agglutinins appeared following the 2nd injection, and after 4 doses agglutination of the Hofer strain was positive in a dilution of 1:3000.

Perez reported the production in broth of a peculiar odor, developing more or less strongly on incubation, which he used for the identification of his bacillus. With many of our cultures no odor was produced in the original broth. In addition, numerous observations lead us to believe that there exists a small group of bacilli closely allied to Perez', but characterized by motility, and a lack of odor in broth cultures. Also, these strains are unaffected by the Perez agglutinins. Otherwise they appear culturally identical. All of our strains which were admitted to the Perez group, developed this odor, tho it appeared at times to be transient in character. The nature of this decomposition was studied in a few instances. Shurly<sup>23</sup> believed that the chemical character of the secretions and the histologic elements present determined the character of the decomposition. I consider that this stench is produced by the activity of a specific proteolytic enzyme. To test this property Perez used serum broth; Hofer, broth; Guggenheim, albuminized media. In order to gauge this activity, tests were made for the presence of methyl-mercaptan, employing the isatin

<sup>23</sup> Diseases of the Nose and Throat, 1907, p. 376.

sulfuric acid method.<sup>24</sup> Flasks of plain broth were inoculated severally with pure cultures of Perez' bacillus, proteus, diphtheroid, Abel's bacillus, and several others. Five cultures of Perez' bacillus produced in broth a strong hydrogen sulfid and mercaptan content; 4 cultures of Abel's organism produced no hydrogen sulfid and no mercaptan; 4 cultures of *B. proteus* produced strong hydrogen sulfid and strong mercaptan; all other tests were negative. These results suggest that the character of the ozena odor is determined by the presence of some of the well-known organic sulfur compounds, especially the mercaptans.

The streptococci appear in the table 5th in order. No special consideration will be given them, since they rarely occurred as the predominating type in the original microscopic pictures.

Sixth in order of isolation stands the proteus group. Just how important this ubiquitous saprophyte may be, it is hazardous to state. Other workers have neglected it entirely. Hajek's organism probably belonged to this group.

It is a luxuriant saprophyte and produces a larger growth and more active enzymic changes in all protein solutions than does Perez' bacillus. It also produces a most nauseating odor in pure culture. This odor is to be distinguished from that produced by other organisms by its suggestive mouselike quality.

#### PATHOGENIC ACTION OF PEREZ' BACILLUS

The work reported in the last division of this paper verifies the finding of Perez and Hofer. They proved that this bacillus was pathogenic for laboratory animals; large doses produced death in rabbits, small doses produced marked reaction, seen in the nasal membranes and in the production of excessive mucopurulent secretions, sometimes developing into a chronic condition.

In this study, 28 healthy rabbits received intravenous injections of the Perez culture; 7 rabbits received cultures of *B. proteus*; 6 of Abel's bacillus, together with 3 cultures of an organism known as *Bacillus bronchisepticus*, these last serving as controls.

Of the 28 animals injected with Perez' bacillus 12 died—7 after 24 hours, 1 after 3 days, 2 after 3 weeks, and 2 after 4 weeks. All showed a rise of temperature, increased nasal discharge, and in those animals dying last, caking about the nostrils. Corrosions at the site of inoculation frequently developed, and in the chronic cases emaciation was marked.

The records of the animals receiving *B. proteus* injections show that of 7 under test 3 died, at autopsy presenting conditions similar to those observed in the Perez rabbits. After injection all the animals showed pronounced nasal reactions and the organism was recovered from the discharges in 2 cases. Those animals receiving the *bronchisepticus* cultures developed in 2 instances all the symptoms of snuffles. This condition lasted for several days, recovery following in both instances. In contrast to the foregoing the rabbits which had received the culture of Abel's bacillus showed no reaction and suffered from no infection.

<sup>24</sup> Bauer: *Ztschr. f. physiol. Chem.*, 1902, 35, p. 346.

Future study offers interesting possibilities of reversing the weight of etiologic evidence which is at present ascribed to Perez' bacillus. One of the conclusions arrived at by Herbert and Nicolle in their study of angina was that Abel's bacillus held an important etiologic relationship to certain infections. They stated that it was pathogenic, capable of producing acute and chronic conditions. Allen<sup>25</sup> has made the repeated observation that the Friedländer bacillus is to be found associated with chronic conditions. Babes,<sup>26</sup> in his study of rhinoscleroma, suggested the pathogenic relationship of this bacillus to that disease. Abel's bacillus, the original Friedländer's bacillus, and Frosch's bacillus are considered by Perkins<sup>27</sup> to represent but varieties of one group, under the title of *B. mucosus-capsulatus*.

If the two organisms are compared on the basis of their pathogenic action in experimental animals, the etiologic importance of the Perez organism seems pronounced. However, when Hofer selected control organisms for his experimental work, he unfortunately chose cultures having practically no value as regards the main point of his work—to prove specific action on the part of Perez' bacillus. His results, together with those of others, would have been much more conclusive had he used organisms found in ozena exudates and infections common to his animals.

It has long been observed that rabbits and other laboratory animals are very susceptible to a form of infection spoken of as "snuffles." Dogs, likewise, are known to succumb to a form of rhinitis presenting symptoms similar to those of ozena in man. In a few reports the idea that the human infection has arisen from the canine is set forth, though no proof has, as yet, been offered in support of this assumption. Recently, Ferry,<sup>28</sup> McGowan,<sup>29</sup> and Torry,<sup>30</sup> working independently, discovered and proved the relationship of a small bacillus known as *B. bronchisepticus* to all cases of canine distemper.

*B. bronchisepticus* is capable of producing in rabbits a type of rhinitis difficult to be distinguished clinically from the pathologic condition produced by Perez' bacillus. In 4 instances rabbits which had received intravenous injections of Perez' bacillus and organisms not Perez' developed "snuffles." From nasal discharges almost pure cultures of *B. bronchisepticus* were isolated. The close cultural characteristics of these two organisms, especially if differential study is not carried out in detail, might be easily overlooked.

<sup>25</sup> The Bacterial Diseases of Respiration, and Vaccines in their Treatment, 1913.

<sup>26</sup> Kolle and Wassermann, Handb. d. pathogen. Mikro-organismen, 1913, 5, p. 1237.

<sup>27</sup> Jour. Infect. Dis., 1904, 1, p. 241.

<sup>28</sup> Ibid., 1911, 8, p. 399.

<sup>29</sup> Jour. Path. and Bacteriol., 1911, 15, p. 381.

<sup>30</sup> Jour. Med. Research, 1913, 27, p. 291.

## FREQUENCY OF OZENA TYPES IN OTHER CONDITIONS

In order to measure the comparative values of the Abel, Perez, and proteus organisms in ozena, a control study was made (in co-operation with Dr. W. H. Price, health officer of Detroit, and Dr. D. M. Griswold, in charge of the laboratories) to determine their presence in other conditions. A bacteriologic survey, including 1,400 examinations of cultures from suspected cases of diphtheria, was made. Attention was given to the relative percentages of cases in which the leading types appeared. The results were as follows:

Abel's bacillus .....	3 %
Perez' bacillus .....	0.1 %
B. proteus .....	1 %

When we contrast with the foregoing the relative percentages of our 50 cases in which these organisms occurred—Abel's bacillus 60%, Perez' bacillus 44%, B. proteus 40%—we find the results agreeing with those obtained by previous observers. Hence the presence of these bacilli in one condition and their absence in other conditions merits serious consideration. It is my opinion that the presence in ozena of Abel's bacillus indicates a pathologic relationship, and I would ascribe to it the rôle of an etiologic factor. When this bacillus has established a foothold in the nasal mucosa, the way is open for the superimposition of the ozena processes, which are dependent on the entrance of a definite species of bacteria.

## CONCLUSIONS

Since in the ozena stage of atrophic rhinitis Löwenberg-Abel's bacillus is found to predominate and since this organism is seldom present in healthy nasal mucosa, a relationship seems to be indicated between this bacillus and the pathologic condition.

Perez' bacillus can be isolated from an important percentage of cases of ozena. It is not present in normal cases or in infections other than ozena, and rarely exists in atrophic rhinitis.

The peculiar ozena odor is due to the presence of volatile products of protein-decomposition, belonging to the group of the organic sulfids, especially the mercaptans.

Such organic sulfids are produced by both Perez' bacillus and B. proteus, but not by Löwenberg-Abel's bacillus.

Perez' bacillus produces acute and chronic conditions in animals and it can be obtained from the nasal membranes of such animals.

*B. bronchisepticus* and *B. proteus* are pathogenic for rabbits and capable of producing the same clinical picture with its variations.

In view of the omission of proper controls, further work is necessary to warrant the full acceptance of Perez' bacillus as the most important etiologic factor in ozena.

# A COMPARATIVE STUDY OF COLON BACILLI ISOLATED FROM HORSE, COW, AND MAN \*

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In the routine analysis of water, to determine its potability, the presence of *B. coli* in 1 c.c. is taken as an index of pollution. The real danger from a polluted or infected water supply is typhoid fever. The colon bacillus is easily determined if present. The determination of the typhoid bacillus is much more difficult. The typhoid organism is never present in a fecally polluted stream or source of water supply without the colon organism's accompanying it. If the colon bacillus is present, the typhoid bacillus is likely to be present also. It is for this reason that the presence of *B. coli* is taken as an index of pollution in determining the potability of water.

The colon organism occurs in the intestines of all warm-blooded animals, and is always present in their excrement. Up to the present time, the colon bacilli in the different animals have not been shown to exhibit any marked or distinguishing features. If some feature or features peculiar to the colon bacilli of one animal could be discovered, it might be of material value in water analysis, as indicating the source of fecal pollution.

Acid-forming streptococci, which are also an index of pollution, have been shown by Winslow and Palmer<sup>1</sup> to exhibit marked differences in their ability to ferment carbohydrates. A summary of this work is given in the following table.

TABLE 1  
COMPARATIVE FERMENTATION POWERS OF STREPTOCOCCI FROM HORSE, COW, AND MAN

Strain	Percentage of Positive Results (300 Strains) in		
	Lactose	Raffinose	Mannite
Human.....	62	6	28
Equine.....	8	4	2
Bovine.....	52	28	6

\* Received for publication January 1, 1916.

<sup>1</sup> Jour. Infect. Dis., 1910, 7, p. 1.

"The rarity of lactose-fermenting streptococci from the horse, makes it probable that this group can be used for distinguishing pollution by street washings from that due to domestic sewage, and the fact that a considerably large proportion of bovine strains ferment raffinose should make it possible to use the ratio to distinguish between washings from pastures and cultivated land from sewage."<sup>2</sup>

Clemesha<sup>3</sup> in India did not obtain these results, however. In addition, the difficulty of isolating the small acid-forming streptococci, in comparison with the ease with which colon bacilli are isolated, is great. The usual media for examining water, litmus lactose agar, would almost entirely eliminate streptococci of equine origin.

With *B. coli*, the fermentation of carbohydrate media offers no such marked means of differentiation between species or strains of colon bacilli from different animals, altho it serves to break the colon group into separate and distinct organisms.

If a quantitative view were taken in the study of these colon organisms, some good results might accrue and serve as a means of differentiation between the colon bacilli of different origins. The acid-producing properties of these organisms in different carbohydrate media have been worked with, to discover whether the actually measured acid produced, or the quantitative aspect, would offer any means of distinguishing between equine, human, and bovine colon bacilli. The statistical method was first used in this field by Andrews and Horder<sup>4</sup> in 1906 in their work with streptococci. In this country it was first used in 1908 by Winslow and Winslow,<sup>5</sup> in their systematic study of the coccaceae.

Altho the original aim of this work has not been realized, some very interesting and useful information has been obtained through the study.

#### HISTORICAL REVIEW

The fermentation of the different carbohydrate media and allied substances by the colon group, has been studied with a view to separating the different members of the group and classifying them by means of the fermentation reactions. Büchner<sup>6</sup> in 1885 was the first to note the production of acid and gas in sugar broth inoculated with a colon organism. The real work began with Durham<sup>7</sup> in 1900. It has since been increased and augmented by the work in England of Houston<sup>8</sup> and MacConkey,<sup>9</sup> and in this country by the work of Winslow

<sup>2</sup> Prescott and Winslow: *Elements of Water Bacteriology*, 1913, p. 210.

<sup>3</sup> *Bacteriology of the Surface Waters in the Tropics*, 1912.

<sup>4</sup> *Lancet*, 1906, 171, p. 708.

<sup>5</sup> *The Systematic Relation of the Coccaceae*, 1908.

<sup>6</sup> Cited by Browne, *Jour. Infect. Dis.*, 1914, 15, p. 580.

<sup>7</sup> *Jour. Exper. Med.*, 1900, 5, p. 353.

<sup>8</sup> Cited by Browne, *Jour. Infect. Dis.*, 1914, 15, p. 580.

<sup>9</sup> *Jour. Hyg.*, 1905, 5, p. 353.

and Walker,<sup>10</sup> Graham Smith,<sup>11</sup> Howe,<sup>12</sup> Jackson,<sup>13</sup> Browne,<sup>14</sup> and Rogers, Clark, and Davis,<sup>15</sup>

Durham<sup>16</sup> on the basis of fermentation (acid and gas in carbohydrate) broke up the intestinal organisms into 3 main groups, (1) *B. typhi*, which does not ferment dextrose, lactose or saccharose, (2) *B. enteritidis*, which ferments dextrose, but not lactose, and (3) *B. coli*, which ferments both dextrose and lactose. This was the starting point. Houston<sup>17</sup> and Savage<sup>18</sup> each tried to distinguish between colon bacilli isolated from different animals, but in this they were unsuccessful.

Winslow and Walker,<sup>19</sup> working with 25 strains of human colon bacilli, found acid produced with dextrose, galactose, lactose, maltose, xylose, and dextrin. None fermented inulin or maltose. With raffinose, saccharose, and dulcitol, some caused fermentation while others did not. Smith<sup>20</sup> measured the acid produced in different sugars by *B. coli*, and Howe's<sup>21</sup> results with 641 strains of coli from 21 men, 540 of which were nonliquefiers, agreed with Smith's in that the sugars formed a metabolic gradient. He came to the conclusion that gas-production was a poor criterion and that acid-production was a sound one. He also broke the organisms up into groups, according to their power of fermenting or not fermenting the test substances. The last step was carried much farther by Jackson,<sup>22</sup> who broke the so-called colon group up into 4 distinct organisms—*B. communior*, *B. communis*, *B. aerogenes*, and *B. acidi-lactici*—by means of 4 test substances, dextrose, lactose, dulcitol, and saccharose. *B. communior* is dextrose +, lactose +, dulcitol +, saccharose +. *B. communis* is dextrose +, lactose +, dulcitol +, saccharose —. *B. aerogenes* is dextrose +, lactose +, dulcitol —, saccharose +. *B. acidi-lactici* is dextrose +, lactose +, dulcitol —, saccharose —.

Rogers and his co-workers,<sup>23</sup> who published papers dealing with the fermentation powers of colon organisms isolated from cows, were especially interested in the gas-production and the relation of the hydrogen to the carbon dioxide. They also measured the acid produced in carbohydrates but drew no definite conclusions from this part of the work. They obtained rather high percentages of acid. None of the 150 strains fermented inulin.

Browne<sup>24</sup> studied several types of human coli, some isolated from polluted waters at Narragansett Bay, R. I., some isolated from Italian immigrants, and some isolated from laboratory assistants. He found that the maximal acid-production took place in 24 hours at the optimal temperature of 37.5 C.; that the most favorable medium contained 1% of the test substance; and that the amount of acid produced was fixed by the organism's toleration of acid. There is an end point for acid-production for each organism. This was confirmed by

<sup>10</sup> Science, 1903, 17, p. 797.

<sup>11</sup> Cited by Browne, Jour. Infect. Dis., 1914, 15, p. 580.

<sup>12</sup> Science, 1912, 35, p. 225.

<sup>13</sup> Jour. Am. Pub. Health Assn., 1911, 1, p. 938.

<sup>14</sup> Jour. Infect. Dis., 1914, 15, p. 580.

<sup>15</sup> Ibid., 1914, 14, p. 411.

<sup>16</sup> Prescott and Winslow: Elements of Water Bacteriology, 1913, p. 93.

<sup>17</sup> Ibid., p. 141.

<sup>18</sup> Ibid.

<sup>19</sup> Science, 1907, 26, p. 797.

<sup>20</sup> Centralbl. f. Bacteriol., 1895, 18, p. 494.

<sup>21</sup> Science, 1912, 35, p. 225.

<sup>22</sup> Jour. Am. Pub. Health Assn., 1911, 1, p. 938.

<sup>23</sup> Jour. Infect. Dis., 1914, 14, p. 411; 15, p. 99.

<sup>24</sup> Ibid., p. 580.

Kligler.<sup>25</sup> If the initial reaction is alkaline, more acid is produced than if the reaction is neutral. Less is produced if the medium is acid.

This in a brief way sums up the work that has been done in regard to the fermentation of carbohydrate media and allied substances by the colon group of organisms.

#### COLLECTION OF MATERIAL

The bovine and equine colon bacilli used were isolated from feces, the human colon bacilli from raw sewage. One hundred samples of horse manure were collected from the streets of West Lafayette and from the waiting stables in Lafayette. The samples of cow feces were secured from about 10 different farms within a radius of 10 miles of Lafayette. The sewage from which the human strains were isolated was obtained at the opening of the West Lafayette sewer into the Wabash river. Samples were collected on several different days and at different times to insure different strains. As the ground was hard and frozen at the time, neither the street washings nor the leachings from pasture lands got into the sewage; it was all household waste.

#### ISOLATION OF THE ORGANISMS

In no case were more than 2 strains of colon bacilli isolated from a sample of sewage or from a sample of feces, and usually but one was taken from the sample.

The isolation was accomplished with about the same procedure in each case but with a variety of culture media. A small amount of feces was inoculated into sterile lactose peptone bile (1% lactose, 1% peptone). After 24 hours at 37.5 C. a platinum loopful of this material was plated with litmus lactose agar and again incubated for 24 hours at 37.5 C. Then typical colonies were picked from the plates and streaked on litmus-lactose-agar plates. After 24 hours' growth at 37.5 C., typical colonies were inoculated on agar streaks. These served as the stock cultures for the work.

At times, instead of using lactose peptone bile, broth was used in conjunction with litmus lactose agar. Good results were obtained by substituting endo media in the place of litmus lactose agar, endo media being a lactose agar to which fuchsin has been added, the whole being then decolorized with sodium sulfite.

The action of the bile salts in the bile inhibits the development of the saprophytic bacteria, but does not inhibit either typhoid or colon bacilli. On litmus lactose agar, the colon organisms give rise to typical red colonies, due to the production of lactic acid. With endo media, brick-red colonies develop in the case of colon bacilli, due to the production of lactic acid, which neutralizes the sodium sulfite, thus allowing the fuchsin to regain its color.

#### TECHNIC

Seven different carbohydrates were worked with. One monosaccharid, dextrose; 2 disaccharids, lactose and saccharose; 1 trisaccharid, raffinose; 1 hexatomic alcohol, mannite; 1 glucosid, salicin; and 1 starch, inulin.

One percent of each of these test substances was added to the sugar-free broth, made according to the standard directions of the American Public Health Association. Three grams of Liebig's extract per liter were used instead of chopped beef. The muscle sugar present in the broth was eliminated by inoculating the sterile infusion of meat extract and distilled water, with a virulent culture of *B. coli* and incubating at 37.5 C. for 24 hours. This destroyed the

<sup>25</sup> Jour. Infect. Dis., 14, p. 81.

muscle sugar by fermentation. The material was then made into standard broth and sterilized. Usually 7 liters of material were made up at one time.

The amount of broth necessary for one test substance (usually 1 liter) was measured out and 1% of the test substance added. The reaction was adjusted as nearly as possible to the neutral point, phenolphthalein being used as an indicator.

About 8 c.c. of each test medium were run into test tubes, 105 in all, and sterilized. The media were then ready for inoculation.

One set of strains was worked with at a time. The tubes were inoculated with the organisms from a 24-hour-old broth culture, by means of a standard platinum loop. They were then incubated at 37.5 C. for 24 hours; then titrated with N/20 NaOH and the amount of acid produced in each individual case recorded. Controls, prepared at the same time, were tested for sterility and for the initial reaction of the media. Five cubic centimeters of the substance were run into 45 c.c. of distilled water contained in a 150-c.c. Erlenmeyer flask. Phenolphthalein was added and the titration proceeded with.

In addition to the acid-production in the different test substances, a few other characters were studied; e. g., the gram stain, gelatin-liquefaction, and indol-production.

The 100 strains of colon bacilli of each type caused no liquefaction of gelatin within a week, were all gram-negative, and all productive of indol in peptone solution. The results with respect to acid-production by human, bovine, and equine colon bacilli are given in Table 2.

TABLE 2  
ACID-PRODUCTION BY COLON BACILLI  
PERCENTAGE OF ACID IN TERMS OF NORMAL NaOH

Strain	Dextrose	Mannite	Salicin	Lactose	Saccharose	Raffinose	Inulin
A. BY HUMAN COLON BACILLI							
1	2.6	2.3	2.0	2.3	1.6	1.2	—
2	2.5	2.7	—*	2.4	—	—	—
3	2.6	2.5	2.5	2.4	2.6	1.7	—
4	2.5	2.5	2.5	2.2	2.3	1.3	—
5	2.4	2.8	2.1	2.2	2.0	2.0	—
6	2.6	2.4	1.6	2.4	2.7	1.5	—
7	2.6	2.4	—	2.8	1.0	1.6	—
8	2.5	2.4	1.6	2.1	—	1.6	—
9	2.7	2.1	—	2.6	2.4	—	—
10	2.8	2.5	3.8	3.1	2.3	1.4	—
11	2.3	2.4	—	2.0	—	1.6	—
12	2.9	2.6	—	2.8	1.1	—	—
13	2.4	2.7	—	2.1	—	1.2	—
14	2.6	2.7	—	2.4	—	—	—
15	2.6	2.5	—	2.1	—	2.2	—
16	2.7	2.5	1.8	2.3	2.6	1.0	—
17	2.6	2.3	2.2	2.5	2.5	2.3	—
18	2.6	2.6	1.9	2.4	2.3	1.2	—
19	2.8	2.6	1.2	2.4	—	—	—
20	2.6	2.6	—	2.4	2.9	1.3	—
21	2.6	2.8	1.8	2.2	—	—	—
22	2.6	2.3	2.6	2.2	2.3	1.7	—
23	2.7	2.4	1.8	2.2	1.4	1.1	—
24	2.4	2.4	—	2.2	1.3	1.2	—
25	2.6	2.3	—	2.0	1.1	1.2	—

\* The sign — means no acid produced.

TABLE 2—Continued

ACID-PRODUCTION BY COLON BACILLI  
PERCENTAGE OF ACID IN TERMS OF NORMAL NaOH

Strain	Dextrose	Mannite	Salicin	Lactose	Saccharose	Raffinose	Inulin
A. BY HUMAN COLON BACILLI							
26	2.3	2.2	—	2.2	—	—	—
27	2.5	2.1	—	2.0	2.6	1.5	—
28	2.5	2.2	2.0	2.2	2.8	1.7	—
29	2.7	2.3	2.5	2.3	1.7	1.6	—
30	2.3	2.4	1.7	2.3	—	—	—
31	2.6	2.4	2.5	2.3	2.5	1.5	—
32	2.4	2.4	1.5	2.2	2.6	1.5	—
33	2.4	2.2	2.1	2.3	2.2	1.5	—
34	2.7	2.5	2.6	2.3	2.5	1.8	—
35	2.4	2.4	2.7	2.4	1.6	1.6	—
36	2.6	2.7	3.3	2.3	2.7	1.7	—
37	2.5	2.7	1.0	2.4	2.6	1.6	—
38	2.5	2.5	3.5	2.6	1.3	1.2	—
39	2.5	2.6	3.5	2.5	2.5	1.3	—
40	2.8	2.5	1.9	2.6	2.3	1.5	—
41	2.6	2.3	2.6	2.7	1.2	1.4	—
42	2.4	2.5	2.2	2.3	1.7	1.6	—
43	2.8	2.5	2.5	2.1	2.0	1.2	—
44	2.5	2.5	2.1	2.3	2.8	1.5	—
45	3.1	2.5	1.8	2.3	2.6	1.2	—
46	2.7	2.5	1.9	2.4	2.2	1.3	—
47	2.6	2.5	2.6	2.3	2.5	1.4	—
48	2.7	2.5	1.5	2.3	1.3	1.1	—
49	2.4	2.8	—	2.1	1.1	1.1	—
50	2.6	2.7	—	2.1	2.3	1.3	—
51	2.1	1.7	—	1.9	1.8	2.0	—
52	2.8	2.5	2.8	2.7	1.4	1.7	—
53	2.3	2.6	3.0	2.5	2.0	2.3	—
54	2.4	2.3	2.6	3.0	2.5	1.5	—
55	2.4	2.2	2.4	2.6	2.5	2.0	—
56	3.0	2.2	3.0	4.1	2.5	1.9	—
57	2.5	2.3	2.4	2.2	1.9	1.5	—
58	2.1	2.1	2.3	1.8	1.8	2.2	1.1
59	2.4	2.5	2.1	1.8	2.3	2.3	—
60	3.7	2.1	3.7	4.2	4.0	2.2	—
61	2.4	2.4	2.4	2.0	2.3	2.4	1.2
62	3.5	2.1	2.8	2.3	3.5	3.5	—
63	2.4	2.2	2.5	2.2	2.7	2.3	—
64	3.0	2.2	2.1	2.2	2.4	1.7	—
65	2.6	2.2	2.0	2.3	1.1	1.0	—
66	2.6	2.2	—	2.3	1.6	1.6	—
67	2.4	2.2	1.4	2.3	1.4	1.1	—
68	2.6	2.4	2.1	2.1	2.3	1.8	—
69	2.7	2.3	1.9	2.1	2.5	1.7	—
70	2.6	2.4	—	2.4	1.2	1.6	—
71	2.4	2.1	2.3	2.2	2.3	1.8	—
72	2.3	2.0	2.2	2.2	2.1	2.0	—
73	2.5	2.4	2.2	2.1	2.3	1.7	—
74	2.5	2.1	1.9	4.0	2.3	1.9	—
75	2.6	2.0	2.1	2.1	2.4	2.8	—
76	2.4	1.9	2.6	2.7	2.0	2.7	—
77	2.6	2.0	1.9	1.9	2.3	2.1	—
78	2.5	2.1	2.2	2.2	1.7	1.9	—
79	3.8	2.7	3.3	2.7	2.8	1.7	—
80	2.5	2.3	1.3	2.4	2.8	4.0	—
81	2.5	2.4	1.2	2.1	2.3	1.2	—
82	2.5	2.0	3.7	3.4	4.3	2.4	—
83	2.7	2.3	3.3	2.5	2.2	2.0	—
84	2.6	2.5	2.5	2.5	2.1	1.8	—
85	2.6	2.3	1.5	2.3	1.4	1.3	—
86	2.6	2.7	2.2	2.3	2.3	1.3	—
87	2.5	2.4	1.9	2.2	—	—	—
88	2.4	2.0	2.6	2.1	—	—	—
89	2.6	2.4	2.4	2.2	1.5	1.8	—

TABLE 2—*Continued*  
ACID-PRODUCTION BY COLON BACILLI  
PERCENTAGE OF ACID IN TERMS OF NORMAL NaOH

Strain	Dextrose	Mannite	Salicin	Lactose	Succharose	Raffinose	Inulin
A. BY HUMAN COLON BACILLI							
90	2.6	2.3	2.6	2.3	2.6	2.4	—
91	2.8	2.4	3.3	3.4	2.5	2.1	—
92	3.2	2.3	3.6	2.6	2.3	2.4	—
93	2.4	2.3	2.4	2.2	2.5	2.6	—
94	2.3	2.2	2.3	2.0	2.2	2.4	—
95	2.7	2.3	2.5	2.5	2.4	1.9	—
96	2.2	2.3	2.4	2.6	2.1	2.3	—
97	2.6	2.3	2.0	2.8	2.7	2.3	—
98	2.5	2.0	1.8	2.8	2.3	2.6	—
99	2.6	2.2	1.8	2.2	2.0	—	—
100	2.6	2.2	2.3	2.2	1.2	1.7	—
B. BY BOVINE COLON BACILLI							
1	2.5	2.4	1.6	1.9	—	—	—
2	2.5	2.5	2.4	2.0	1.2	2.1	1.1
3	2.4	2.4	1.8	2.0	1.3	1.7	—
4	2.5	1.9	2.3	2.1	2.4	2.7	1.4
5	2.3	2.4	1.9	1.9	1.5	1.8	1.4
6	2.6	2.5	1.6	1.9	1.8	1.5	1.3
7	2.5	2.5	1.9	1.9	—	1.7	—
8	2.8	2.5	2.1	1.9	1.2	1.6	1.3
9	2.4	2.4	—	2.1	2.4	1.3	—
10	2.6	2.6	—	1.8	1.5	1.9	—
11	2.4	2.4	1.7	2.1	1.2	1.7	—
12	2.8	2.6	1.9	2.0	2.0	2.5	—
13	2.6	2.5	1.6	2.0	1.3	1.8	—
14	2.6	2.3	—	2.1	1.6	1.8	—
15	2.6	2.5	—	2.0	1.2	1.8	—
16	2.5	2.3	2.3	2.0	1.6	1.5	1.4
17	2.8	2.4	2.0	2.0	1.6	1.9	1.0
18	2.8	2.5	2.5	1.9	3.1	1.8	—
19	2.6	2.0	—	2.1	1.7	1.6	—
20	2.4	2.6	1.4	2.1	1.4	2.1	—
21	2.6	2.9	2.2	2.1	2.4	1.8	—
22	2.7	2.9	1.8	2.1	1.4	2.1	—
23	2.6	2.7	—	2.1	—	2.0	—
24	2.7	2.7	—	1.9	1.4	1.8	—
25	2.6	2.1	1.4	1.9	1.6	2.3	—
26	2.4	2.5	1.9	1.9	1.3	1.8	—
27	2.5	2.5	—	1.9	1.4	1.8	—
28	3.0	2.3	2.6	2.4	3.5	2.6	1.4
29	2.6	2.5	1.7	2.0	1.4	1.8	—
30	2.4	2.4	1.8	2.0	—	—	—
31	2.7	2.6	2.0	2.4	2.5	2.4	—
32	3.3	2.5	2.3	2.0	1.4	2.6	—
33	2.7	2.5	—	2.3	—	2.3	—
34	2.6	2.6	1.3	1.7	1.9	—	—
35	2.7	2.4	1.8	2.9	1.4	2.1	—
36	2.8	2.4	—	2.1	—	1.9	—
37	2.6	2.6	2.0	2.2	1.4	—	—
38	2.5	2.7	1.8	1.8	1.3	1.6	—
39	2.7	2.4	2.3	2.0	1.5	2.4	—
40	2.6	2.5	—	1.7	1.7	1.9	—
41	2.8	2.6	1.7	2.1	2.6	2.0	—
42	2.6	2.4	2.2	2.1	1.7	2.0	—
43	2.8	2.5	3.6	2.1	1.7	2.3	—
44	2.7	2.6	—	2.1	1.7	2.0	—
45	2.6	2.4	—	2.1	1.5	2.3	—
46	2.6	2.6	2.4	2.0	1.3	2.6	—
47	2.7	2.3	1.8	2.1	3.0	2.9	—
48	2.5	2.4	2.2	2.1	1.7	2.4	—

TABLE 2—Continued

ACID-PRODUCTION BY COLON BACILLI  
PERCENTAGE OF ACID IN TERMS OF NORMAL NaOH

Strain	Dextrose	Mannite	Salicin	Lactose	Saccharose	Raffinose	Inulin
B. BY BOVINE COLON BACILLI							
49	2.7	2.3	1.8	1.9	2.9	1.8	—
50	3.3	2.1	2.1	2.0	—	2.5	—
51	2.4	2.4	—	1.9	1.6	1.9	—
52	2.5	2.4	1.9	1.9	2.3	1.6	—
53	2.6	2.3	2.1	2.0	3.4	2.8	1.2
54	2.5	2.5	2.2	1.8	2.7	2.3	1.3
55	3.5	2.5	2.8	3.0	2.8	1.8	1.6
56	2.4	2.5	4.1	2.2	1.6	2.0	—
57	2.8	2.3	2.8	1.9	3.1	2.1	1.2
58	2.5	2.5	1.9	2.1	1.4	1.9	—
59	2.5	2.5	2.0	1.9	1.4	2.0	—
60	2.5	2.6	2.9	2.1	1.6	2.1	—
61	2.4	2.5	2.6	1.9	2.6	2.4	1.4
62	2.4	2.8	1.7	2.0	—	—	—
63	3.2	2.6	3.7	2.6	3.6	2.0	—
64	2.8	2.3	3.4	2.7	3.3	2.5	1.3
65	3.7	2.4	2.7	2.3	3.7	2.2	1.5
66	2.7	2.8	1.8	1.7	—	—	—
67	2.6	2.5	2.3	1.6	2.5	2.5	—
68	2.4	2.3	—	1.7	1.5	2.0	—
69	2.6	2.4	2.9	1.8	1.3	1.6	1.2
70	3.4	2.2	2.7	3.2	3.2	2.3	1.3
71	2.6	2.3	3.2	2.0	1.7	2.7	1.4
72	3.0	2.3	2.6	1.8	2.1	2.1	—
73	2.5	2.2	2.8	1.6	1.4	2.4	—
74	2.4	2.3	2.0	2.0	2.6	2.3	1.3
75	2.4	2.4	1.8	1.8	1.6	2.0	—
76	2.7	2.6	2.6	1.7	3.2	2.3	1.3
77	2.6	2.3	2.0	2.1	1.3	1.9	—
78	2.5	3.2	1.5	2.3	1.4	2.6	—
79	2.5	2.4	1.5	1.9	1.1	2.1	—
80	2.6	2.4	1.9	1.9	1.2	2.0	—
81	3.5	2.2	2.9	2.1	3.3	2.2	—
82	2.8	2.6	2.9	2.2	2.7	2.1	2.8
83	2.8	2.6	2.0	1.9	1.9	2.6	—
84	2.7	2.5	—	2.2	1.6	1.1	—
85	3.1	2.6	3.1	2.5	2.4	2.9	—
86	2.6	2.2	—	1.9	—	—	—
87	2.6	2.4	1.8	1.9	1.8	1.8	—
88	2.5	2.4	2.7	2.2	2.3	2.2	1.6
89	2.9	2.4	3.5	2.2	3.0	2.2	—
90	3.0	2.3	3.1	2.0	1.8	2.1	—
91	2.6	2.5	1.5	1.9	1.5	2.2	—
92	2.5	2.8	1.4	1.9	1.7	2.0	—
93	2.5	2.5	1.5	2.1	1.5	2.0	—
94	2.5	2.2	1.9	1.8	1.5	2.0	—
95	2.5	2.3	—	2.0	1.5	2.3	—
96	2.9	2.4	2.3	1.9	2.9	2.0	1.3
97	2.7	2.4	1.7	1.9	1.4	2.2	—
98	2.7	2.4	1.4	2.0	1.6	2.0	—
99	2.5	2.2	1.8	2.0	3.0	2.4	—
100	2.6	2.6	2.0	1.9	1.2	2.1	—

C. BY EQUINE COLON BACILLI

	Dextrose	Mannite	Salicin	Lactose	Saccharose	Raffinose	Inulin
1	2.6	1.6	2.0	2.1	2.3	1.5	—
2	2.7	2.2	1.4	2.1	1.3	1.7	—
3	2.5	1.9	1.6	2.1	—	1.8	—
4	2.5	2.1	2.1	2.1	2.7	1.5	—
5	2.4	1.9	1.7	2.1	1.3	1.6	—
6	2.7	2.0	—	1.9	2.1	1.4	—
7	2.9	2.1	3.0	2.1	2.3	1.6	—

TABLE 2—Continued

## ACID-PRODUCTION BY COLON BACILLI

## PERCENTAGE OF ACID IN TERMS OF NORMAL NaOH

Strain	Dextrose	Mannite	Salicin	Lactose	Saccharose	Raffinose	Inulin
C. BY EQUINE COLON BACILLI							
8	2.4	1.9	—	2.0	1.3	1.5	—
9	2.5	2.1	—	2.0	1.0	1.6	—
10	2.5	2.1	2.0	2.1	3.4	1.7	—
11	2.5	2.0	—	2.1	1.3	1.3	—
12	2.7	2.2	1.8	2.1	2.0	1.5	—
13	2.6	2.1	1.8	2.1	—	—	—
14	2.6	2.0	—	2.4	1.2	1.6	—
15	2.5	2.1	—	2.3	1.2	1.5	—
16	2.5	2.3	—	1.9	1.6	1.9	—
17	2.5	2.2	—	2.1	1.3	1.6	—
18	2.2	2.2	—	2.1	2.4	1.5	—
19	2.7	2.1	2.3	2.1	2.5	2.3	—
20	2.6	1.8	2.4	2.1	1.6	2.0	—
21	2.5	2.0	1.7	2.0	1.4	1.8	—
22	2.4	2.0	1.8	2.1	2.1	1.5	—
23	2.5	2.0	1.6	2.1	1.4	1.9	—
24	2.6	2.0	1.5	2.1	1.3	1.8	—
25	2.4	2.2	—	2.3	1.3	1.5	—
26	2.7	1.9	1.5	2.1	1.3	1.7	—
27	2.3	2.2	—	2.1	1.8	1.6	—
28	2.6	2.0	—	2.2	2.5	1.5	—
29	2.4	2.0	—	2.1	1.3	1.5	—
30	2.4	2.0	1.4	2.1	1.2	1.6	—
31	2.4	2.0	—	2.1	1.3	1.7	—
32	2.5	1.8	1.4	2.1	1.2	1.6	—
33	3.0	2.1	3.4	2.5	4.1	1.6	—
34	2.6	2.1	—	2.0	1.3	1.6	—
35	3.1	2.0	3.1	2.4	4.5	1.7	—
36	2.5	2.3	—	2.3	1.2	1.6	—
37	2.4	2.0	1.8	1.9	—	1.6	1.6
38	2.6	2.4	—	2.1	1.3	1.9	—
39	2.9	2.2	1.9	2.1	—	1.2	—
40	2.7	2.0	—	2.2	1.6	1.5	—
41	2.6	2.0	1.8	1.9	2.7	1.6	—
42	2.4	2.1	1.8	2.3	1.3	2.2	—
43	2.7	2.0	—	2.1	—	—	—
44	2.6	2.2	—	1.9	1.1	1.6	—
45	2.7	2.3	1.9	2.1	1.7	1.6	—
46	2.6	2.1	—	1.9	1.2	1.6	—
47	3.1	1.6	2.3	2.0	2.4	2.0	—
48	2.5	2.0	1.8	1.9	2.7	1.7	—
49	2.8	2.1	3.2	2.5	2.7	1.5	—
50	2.4	2.3	—	2.1	1.2	1.7	—
51	3.1	2.1	2.8	2.8	3.8	1.2	—
52	2.4	1.6	2.0	2.0	1.5	1.8	—
53	2.6	2.3	1.9	2.1	1.3	1.6	—
54	2.4	2.2	1.2	2.1	1.0	1.8	—
55	2.6	1.9	1.5	2.3	3.0	1.9	—
56	2.9	1.9	3.4	2.7	2.6	1.8	—
57	2.9	1.0	2.4	2.7	1.9	1.7	1.0
58	3.4	2.2	3.1	2.9	4.2	1.3	—
59	2.6	2.2	1.2	2.1	1.2	1.4	—
60	3.6	2.1	3.6	1.9	3.8	1.8	—
61	2.4	2.0	—	2.0	—	—	—
62	2.6	2.0	1.6	2.1	1.2	2.0	—
63	2.7	2.2	1.4	1.9	1.4	1.4	—
64	2.8	2.3	2.2	2.4	1.6	1.6	—
65	2.4	2.2	1.9	2.1	—	—	—
66	4.4	2.3	3.0	2.6	2.0	1.3	—
67	2.7	2.1	1.7	2.0	3.3	1.7	—
68	2.6	2.2	—	2.1	1.2	1.5	—
69	4.3	2.1	3.0	3.0	4.1	1.6	—
70	2.5	2.0	1.6	2.2	2.3	1.8	—
71	2.7	1.6	—	2.1	1.5	1.8	—

TABLE 2—*Continued*  
ACID-PRODUCTION BY COLON BACILLI  
PERCENTAGE OF ACID IN TERMS OF NORMAL NaOH

Strain	Dextrose	Mannite	Salicin	Lactose	Saccharose	Raffinose	Inulin
C. BY EQUINE COLON BACILLI							
72	2.4	2.1	1.2	2.1	—	1.5	—
73	2.4	1.9	1.2	2.0	1.3	1.8	—
74	4.2	2.1	3.3	3.2	4.0	1.4	—
75	3.2	2.0	3.6	2.1	1.4	1.6	—
76	2.7	2.4	1.6	2.0	1.3	1.8	—
77	2.9	2.1	2.6	2.1	4.2	1.2	1.0
78	2.5	2.3	1.7	1.8	1.1	1.3	—
79	2.7	2.4	1.1	2.1	2.4	1.4	—
80	2.6	2.0	2.6	2.0	—	1.5	—
81	3.6	2.4	3.4	2.2	3.9	1.7	—
82	3.0	2.4	—	2.1	3.0	1.9	—
83	2.7	2.3	1.7	2.0	1.2	1.5	—
84	2.6	2.2	—	1.6	—	1.8	—
85	3.1	3.0	1.7	2.1	1.3	2.0	—
86	2.8	1.7	2.2	2.6	2.3	1.5	—
87	4.2	1.8	2.1	2.8	2.9	2.0	—
88	2.6	2.3	1.5	2.2	1.3	1.7	—
89	2.6	2.0	1.4	2.0	1.0	2.0	—
90	2.4	2.1	1.5	2.1	—	—	—
91	2.7	2.3	1.5	2.2	1.2	1.6	—
92	3.3	2.0	1.9	2.1	1.6	1.8	—
93	2.5	2.1	1.8	2.0	1.1	1.2	—
94	2.6	2.1	1.5	2.1	1.3	1.7	—
95	4.0	1.9	3.2	3.6	2.7	2.5	1.1
96	2.8	2.2	1.5	1.9	1.3	1.3	—
97	3.6	2.0	3.8	2.1	4.2	1.5	—
98	1.1	2.3	—	2.1	1.4	—	—
99	3.3	2.1	3.1	2.6	4.4	1.4	—
100	2.7	2.3	1.9	2.0	2.3	1.5	—

#### THE ANALYSIS OF THE DATA

The foregoing work may be analyzed according to the following topics:

(1) The number of organisms of each type that produce acid from the test substances, that form indol, that do not liquefy gelatin, that are gram-negative.

(2) The average acid-production by each of the 100 strains of colon bacilli of each type.

(3) The relation between the complexity of the substance fermented and the acid produced.

(4) The relation between the average acid-production and the sugars (dextrose, lactose, saccharose, raffinose) attacked.

(5) The number of strains in each type that produce a given amount of acid and their relation to the substance fermented.

(6) The relation between the average amount of acid produced in each test substance and the origin of the organisms.

TABLE 3  
PERCENTAGE OF ORGANISMS ATTACKING THE VARIOUS SUBSTANCES

Substance	Human	Bovine	Equine	Average
Dextrose.....	100	100	100	100
Mannite.....	100	100	100	100
Lactose.....	100	100	100	100
Raffinose.....	89	93	95	92
Saccharose.....	88	90	91	90
Salicin.....	82	82	73	79
Inulin.....	2	24	4	10
Gelatin (—).....	100	100	100	100
Gram stain (—).....	100	100	100	100
Indol (+).....	100	100	100	100

TABLE 4  
THE AVERAGE ACID-PRODUCTION IN PERCENTAGE OF NORMAL NaOH

Substance	Human	Bovine	Equine	Average
Dextrose.....	2.57	2.67	2.74	2.66
Mannite.....	2.31	2.43	2.08	2.27
Lactose.....	2.38	2.04	2.13	2.18
Saccharose.....	2.18	1.95	1.94	2.02
Salicin.....	2.32	2.16	2.13	2.20
Raffinose.....	1.76	1.88	1.63	1.76
Inulin.....	—	—	—	—

TABLE 5  
THE RELATION BETWEEN THE AVERAGE AMOUNT OF ACID PRODUCED AND THE SOURCE OF THE ORGANISM

Test Substance	Source	Average Acid-Production
Dextrose.....	{ Horse..... Cow..... Man.....	2.74% 2.67 2.56
Mannite.....	{ Cow..... Man..... Horse.....	2.43% 2.31 2.08
Lactose.....	{ Man..... Horse..... Cow.....	2.38% 2.13 2.04
Saccharose.....	{ Man..... Cow..... Horse.....	2.19% 1.95 1.94
Salicin.....	{ Man..... Cow..... Horse.....	2.34% 2.15 2.13
Raffinose.....	{ Cow..... Man..... Horse.....	1.88% 1.76 1.63
Inulin.....	{ Cow..... Horse..... Man.....	— — —

From Table 3 it is evident that all the strains studied give the typical reactions of *B. coli* with gelatin, with the gram-stain, and also with regard to the production of indol. The different types agree in that all produce acid in dextrose, lactose, and mannite. The outstanding result of the tests with the other sugars is that a number of the strains of bovine origin produce acid in inulin broth.

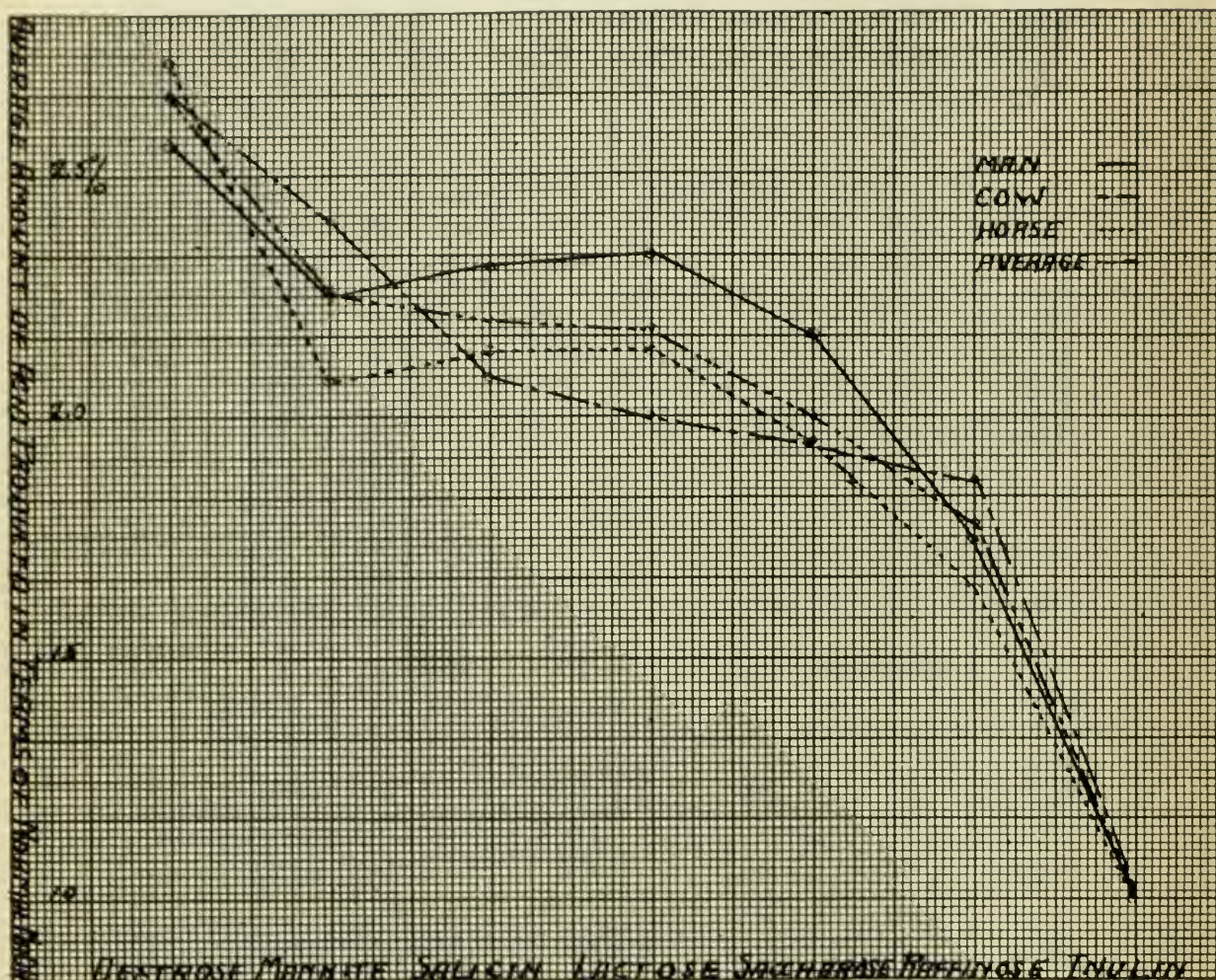


Chart 1. Average acid-production in the test substances.

In general, according to these data, the average amount of acid produced in the different test substances varies from 2.67% to 1.63%. The greater amount of acid is usually produced in substances of lesser complexity. For example, on the average there is more acid produced in dextrose, less in mannite, less in lactose, less in saccharose, less in raffinose, and less in inulin. Salicin and mannite occupy a variable position. When considering the sugars alone—dextrose, lactose,

saccharose, and raffinose—a true metabolic gradient is formed in each of the 3 types and also in the average of the 300 strains taken together. These relations are shown in Charts 1 and 2.

From a perusal of the data it is seen that the acid-production, for the greater part, lies within narrow limits in the case of dextrose, lactose, and mannite. These substances were fermented by every

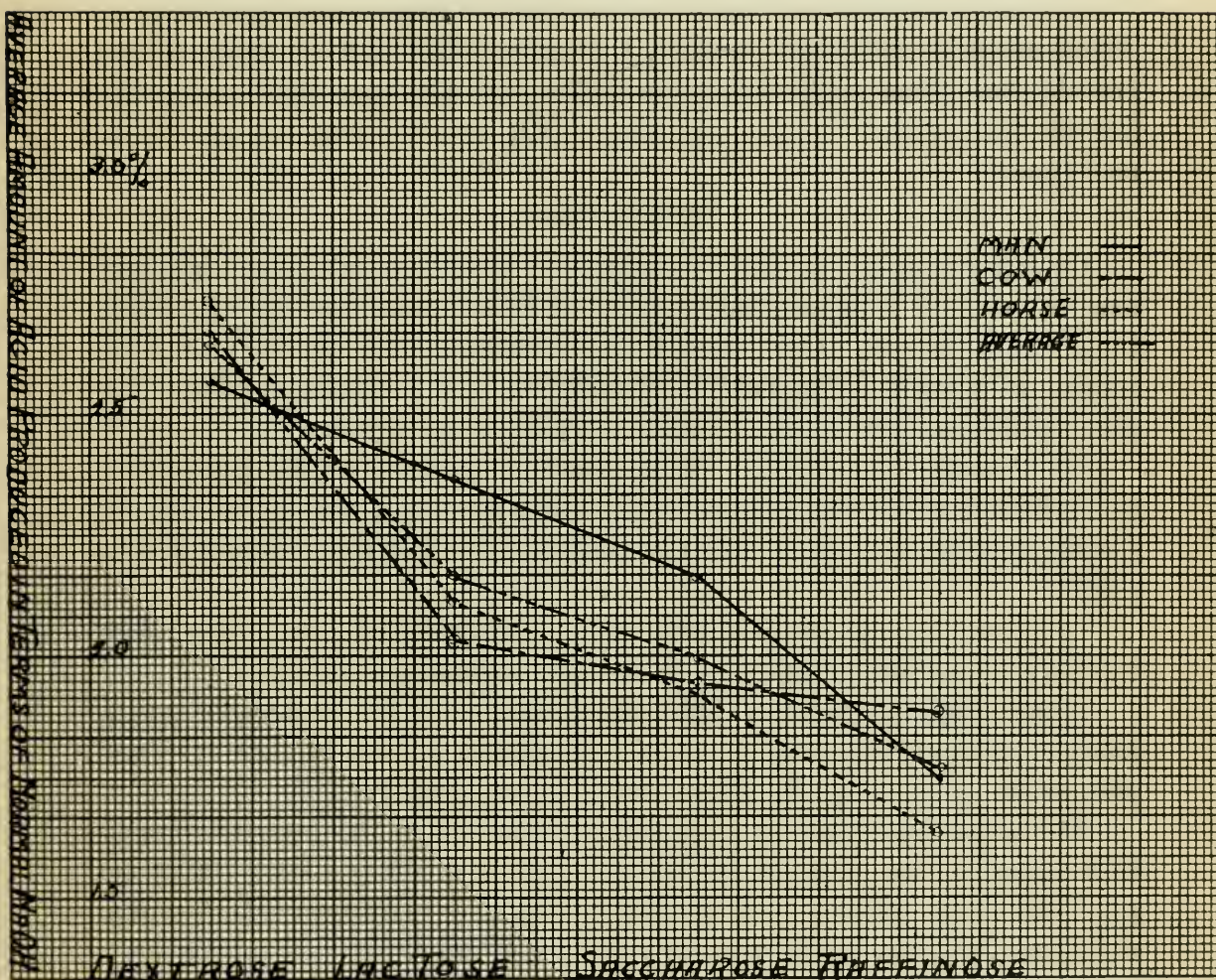


Chart 2. Average acid-production in the sugars.

strain. The larger number of strains in each case produce a certain amount of acid, and the others lie near this point.

With raffinose and saccharose acid-production is not, generally speaking, confined to narrow limits but is spread out. In the case of salicin curves, the acid-production is spread over a very large area.

In the case of dextrose, lactose, and mannite, the high points of acid-production lie close together. In the case of dextrose the high

point of acid-production was the same with human, bovine, and equine colon bacilli, namely, 2.6%; in the case of mannite, it was, human, 2.4%, bovine, 2.4% and equine 2.1%; in the case of lactose it was, human, 2.3%, bovine, 1.9%, and equine, 2.1%.

From the data, it is evident that the average percentages of acid produced by the 3 different types of colon bacilli, do not lie far apart in the case of any given test substance.

It is also evident that the average acid-production does not serve to differentiate between the strains of colon bacilli. For example, in one case bovine colon bacilli head the list, in another case they are at the bottom, in another in the middle. This is also true of the other two types.

#### CONCLUSIONS

On an average, the different types of strains—human, bovine, and equine—exhibit a remarkable similarity in all reactions tested, chiefly in acid-production. One remarkable exception was the ability of 24 strains of bovine colon bacilli to produce acid in inulin media. The other differences were not marked enough to be of value.

In all cases the average acid-production for each of the 100 strains of each type resembles that of every other one, and also resembles the average acid-production of all the strains taken together irrespective of origin.

In general, the average amount of acid produced by each type tends to decrease as the complexity of the test substance increases.

A metabolic gradient is definitely shown with the sugars, dextrose, lactose, saccharose, and raffinose. It is clearly shown in each case and also in the average of the three types taken together.

The organisms show a preference for some of the test substances. For example, the average for all the organisms shows that 100% act upon dextrose, lactose, and mannite; 90% on saccharose; 92% upon raffinose; 79% upon salicin; and 10% upon inulin.

With mannite, dextrose, and lactose, the organisms have a high point of acid-production at which the larger percentage of the strains belong. The other strains for the greater part lie immediately on either side of this high point. The acid-production for the larger number is confined to narrow limits.

The high points of acid-production do not lie far apart with dextrose, lactose, and mannite. They coincide in the case of mannite.

In general with saccharose, raffinose, and salicin, this high point is neither clearly shown nor definitely marked. The acid-production varies greatly and is spread over a large area.

## IMMUNE REACTIONS IN SCARLET FEVER \*

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Some time ago<sup>1</sup> we reported the results of anaerobic cultures in scarlet fever. Cultures were made from the throat, blood, lymph glands, kidneys, and urine. This work indicated that during the acute stage of scarlatina a variety of organisms enter the blood and tissues and are excreted, in part at least, through the kidneys. It was not clear from our animal experiments whether any of these organisms caused scarlet fever or whether all existed only as accidental or secondary invaders. Some of the animals died with suspicious rashes, but nothing was obtained which could be called an experimental scarlet fever.

Before studying further the significance of the organisms obtained in cultures, it seemed desirable to test the various fluids from which they were grown in order to determine whether or not a virus or antigen was present that perchance is ultramicroscopic, or which fails to grow with the cultural methods in use. If such could be demonstrated, the secondary importance of the organisms described would seem to be clear.

In testing for the presence of such a virus or antigen, the immune reactions were used. In addition, the blood serum was tested as to toxicity for animals and for the presence of living virus by means of potassium tellurid. The agglutination test had been used with some of the organisms isolated and had proved so unsatisfactory that in the work reported here, the complement-fixation and cutaneous reactions only were used. The antigenic properties of the blood, the throat and mouth secretion, extracts of spleen, and extracts of lymph glands were examined. The results follow.

### BLOOD

*Complement-Deviation Test.*—Antigens in the body fluids have been found by means of complement-fixation in a number of instances. For example, tuberculous antigens were demonstrated in urine and pleuritis

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<sup>1</sup> Dick, G. F. and G. R.: Jour. Infect. Dis., 1914, 15, p. 85.

exudates with known tuberculous antiserum.<sup>2</sup> We have tested antigenic properties of 6 sera by complement-fixation.

These sera were obtained from scarlatinal patients at the height of intoxication as indicated by the temperature, rash, etc. Antisera for these tests were obtained from 8 convalescent scarlet-fever patients at periods varying from the end of the 1st to the end of the 6th week. Sheep erythrocytes and anti-sheep rabbit serum with fresh guinea-pig serum as complement were used. For each test the anti-sheep rabbit serum was titrated with a constant amount of guinea-pig serum, and 0.05 c.c. of complement and 2 units of rabbit amboceptor used. Increased power of hemolysis (both amboceptor and complement) in scarlatinal serum has been described by a number of observers, notably Moro.<sup>3</sup> In this work, in order to guard against interference with fixation by such hemolysins in the serum used as antigen, each serum was divided into 3 parts, each one of which was used for a complete reaction. In the 1st series, the active serum was used as antigen; in the 2nd series, serum heated at 56 C. for one-half hour; in the 3rd series inactivated serum, from which the sheep amboceptor had been removed through absorption by sheep cells. In order to correct the increased power of hemolysis for sheep cells in the convalescent serum, the hemolytic power of the serum was titrated, and, if hemolysin was present, it was inactivated. If the inactivated serum showed an excess of amboceptor, the amboceptor was extracted by excessive amounts of sheep cells. The amount of convalescent antiserum used was 0.1 c.c. in all the tests. The quantities of antigen, that is, serum of early scarlet fever, used were: 0.025 c.c., 0.05 c.c., 0.1 c.c., 0.15 c.c., 0.2 c.c., 0.3 c.c., and 0.5 c.c. The amount of sheep cells used was 0.5 c.c. of a 5% suspension. For each test a control antiserum was used which had been obtained from convalescent diphtheria patients who gave no history of scarlet fever. All the sera used proved negative in Wassermann tests with acetone-insoluble beef-heart-extract antigen.

In all the various ways in which the tests were carried out there was no evidence of complement-fixation. The diphtheria controls were also negative. Kappel<sup>4</sup> obtained negative results in testing the sera of 20 patients in the acute stage of scarlet fever for the presence of antigen by means of complement-fixation. He used only inactivated serum without correction for increased natural amboceptor, so that the test was probably not so delicate as those described here. Results of both methods, however, indicate that complement-fixation fails to demonstrate any specific antigen in the serum of scarlatinal patients as early as the 2nd day of the disease.

*Toxicity of the Serum of Scarlatinal Patients.*—That there is a difference between normal serum and the serum of patients sick with scarlet fever was shown by Hamilton<sup>5</sup> on *Peramœcium*, Syrensky,<sup>6</sup> and more recently also by Bankowski and Szymanowski.<sup>7</sup>

<sup>2</sup> Delse and Parap: *Compt. rend. Soc. de biol.*, 1911, 71, p. 228.

<sup>3</sup> *Wien. med. Wchnschr.*, 1908, 8, pp. 3, 39, 159.

<sup>4</sup> *Wien. klin. Wchnschr.*, 1911, 24, p. 1295.

<sup>5</sup> *Jour. Infect. Dis.*, 1903, 1, p. 211.

<sup>6</sup> *Ztschr. f. Immunitätsf.*, 1914, 20, p. 543.

<sup>7</sup> *Ibid.*, 1913, 16, p. 330.

The last two observers found the serum of scarlatina patients fatal for guinea-pigs in doses of from 0.13 c.c. to 0.4 c.c. per 100 grams' weight of guinea-pig. The serum of normal individuals was fatal for guinea-pigs in doses of from 0.5 to 0.6 c.c. per 100 grams' weight of guinea-pig. Syrensky allowed the blood that was to be tested for toxicity to stand for from 6 to 12 hours before injection. Bankowski and Szymanowski do not mention this interval. Mita and Ito<sup>8</sup> show that after one-half hour the toxicity of foreign serum for guinea-pigs decreases; so that after from 6 to 24 hours it becomes only a third as toxic as when freshly drawn.

In the following experiments blood was collected in centrifuge tubes and the serum separated and injected into guinea-pigs intravenously within one-half hour after withdrawal from the patient. Sera from 2 mildly sick scarlet fever patients were compared with sera from 2 convalescents. The minimal lethal doses of the former were 0.25 c.c. and 0.39 c.c. per 100 grams' weight of guinea-pig. Those of the latter were 0.43 c.c. and 0.5 c.c. per 100 grams.

These results, while less striking than those noted by other observers, indicate that the blood of scarlet-fever patients is more toxic during the time the patient is acutely sick than it is during convalescence. Indeed, from the reports of other observers, the toxicity of acute scarlet-fever serum, unlike normal serum, tends toward an increase on standing rather than a decrease.

*Potassium-Tellurid Test.*—It has long been known that tellurium compounds dissolved in growing cultures of micro-organisms are broken up and the tellurium absorbed by the micro-organisms. King and Davis<sup>9</sup> have shown that for many bacteria potassium tellurid dissolved in the media in the proportion of 1:10,000 to 1:300,000, acts as an efficient indicator of microbic growth. The bacteria absorb the tellurium and are precipitated at the bottom of the culture tube in the form of a black button. Experiments were undertaken to see if any virus could be demonstrated in the serum of scarlet fever which could not be detected by the cultural methods used in the work previously reported.

For this purpose tubes of about 10 c.c. of ascites broth (1:10) containing potassium tellurid were inoculated with 1 c.c. of scarlet-fever serum obtained from acutely sick patients. Six tubes were made for each case of scarlet fever, 3 aerobic and 3 anaerobic, each set containing tellurid in dilutions of 1:20,000, 1:40,000, and 1:100,000. Six similar tubes without serum were used as negative controls in each test. Six positive controls, aerobic and anaerobic, were made by inoculating ascites-broth tellurid dilutions with small gram-negative diphtheroids from the urine of a scarlet-fever patient.

In the sera from 6 cases of scarlet fever there was no precipitation of tellurium more marked than in the negative control tests, which

<sup>8</sup> Ztschr. f. Immunitätsf., 1913, 17, p. 586.

<sup>9</sup> Jour. Pub. Health, 1914, 4, p. 917.

became affected to some extent after about one week's incubation. The positive controls showed marked precipitation in every instance. The test was not so satisfactory for the purpose as might have been hoped from the work of King and Davis,<sup>8</sup> as the differences between positive and negative results, while clear enough for such rapidly growing organisms as they used in their work, were not so clear after 10 days' incubation. Another disadvantage of the test is that it is not adapted to those methods of culture which gave positive results in the work reported previously. The experiments with tellurid are of value only in showing that there probably are no rapidly multiplying organisms present in scarlet-fever serum which grow in ascites tellurid broth.

#### SPLEEN

Extract of spleen for antigen was prepared as follows: The spleen of a child who had died on the 2nd day of scarlet fever—the blood culture during life having been free from streptococci—was ground in a mortar with 0.5% phenol in 0.85% salt solution and the mixture allowed to stand in the ice-box over night. In the morning the supernatant fluid was removed and used as antigen.

*Complement-Fixation.*—Following a method of fixation similar to that described for the tests with blood serum, tests were made with the sera of 6 cases of scarlet fever at various stages, from the 2nd day to the 3rd week of the disease.

The antigen was used in amounts of 0.025 c.c., 0.05 c.c., 0.1 c.c., 0.2 c.c., 0.3 c.c. In 0.3 c.c. the antigen alone inhibited hemolysis, hence no higher strengths were used. The serum was used in the constant quantity of 0.1 c.c. As controls, the antiserum used was tested with acetone-insoluble heart extract, and the antigens with sera of diphtheria patients as antisera. No serum tested showed complement-fixation. Indeed, in some instances the tubes containing scarlet-fever serum and antigen hemolyzed in strengths of antigen which inhibited both alone and with diphtheria serum.

*Cutaneous Tests.*—With the same spleen which was used for complement-fixation, an extract was made with 0.085% salt solution and evaporated at 60 C. to 1/10 its volume; enough phenol then was added to make 0.5%. Before use of the antigen sterility was determined by cultures. In 4 convalescent scarlet-fever patients and 4 diphtheria patients tests made with this antigen similar to the tuberculin cutaneous tests gave uniformly negative results.

#### LYMPH GLANDS

Lymph glands were collected from various parts of the same body from which the spleen just described was obtained. An antigen was

made for complement-fixation by the same method, and fixation tests carried on with the same sera as antisera, that were used with the spleen tests.

The amounts of antigen used were 0.025 c.c., 0.05 c.c., 0.1 c.c., 0.2 c.c., 0.3 c.c., and 0.4 c.c. In the last two quantities the antigen alone gave a partial inhibition. The sera from the scarlet-fever patients were used in 3 ways—active, inactive, and after removal of natural amboceptor by means of washed sheep cells. Controls were used as in the case of the extract of the spleen.

The results of these tests were uniformly negative. Cutaneous tests with lymph-gland extract made in a way similar to those with the spleen extract were negative in 3 cases of scarlet fever and in 3 controls.

The results of complement-fixation tests with organ extracts are in harmony with the results obtained by some observers and at variance with those obtained by others. Following the introduction of the Wassermann test with extract of syphilitic liver as antigen, Haendel and Schultz<sup>10</sup> substituted extract of scarlatinal liver as antigen and obtained 24 positive reactions in 31 cases of scarlatina tested. Uppenheimer<sup>11</sup> obtained negative results with extract of scarlatinal liver as antigen. Sommerfeld,<sup>12</sup> using syphilitic-liver extracts and scarlatinal-liver extracts as antigen, found that some scarlatinal sera fixed complement with both kinds of antigen. There was no relation to the severity of the disease. Scarlatinal serum which fixed with one syphilitic-liver extract failed to do so with other syphilitic-liver extracts, tho both extracts reacted equally well with the sera of syphilitic patients. Hecht, Pelissier, and Wileno<sup>13</sup> obtained negative results in 119 cases in which fixation was tried with alcoholic extracts of scarlatinal liver as antigen. Kolmer<sup>14</sup> tested 250 cases for complement-fixation with alcoholic extracts of both syphilitic and scarlatinal livers and obtained positive results in 2% with syphilitic liver and in 2.4% with scarlatinal liver. In none of the positive cases could syphilis be excluded. He concludes that complement-fixation with scarlatinal liver as antigen and scarlatinal serum as antibody gives negative results. Kappel,<sup>4</sup> testing as antigen the serum, extract of desquamated scales, and watery and alcoholic extract of tonsils of scarlatina, obtained negative results. The Koesslers<sup>15</sup> used extracts of liver, kidney, and lymph glands as antigen.

<sup>10</sup> Ztschr. f. Immunitätsf., 1908, 1, p. 91.

<sup>11</sup> München. med. Wehnschr., 1909, 6, p. 2471.

<sup>12</sup> Arch. f. Kinderh., 1909, 50, p. 38.

<sup>13</sup> Ztschr. f. Immunitätsf., 1909, 2, p. 356.

<sup>14</sup> Jour. Exper. Med., 1911, 14, p. 235.

<sup>15</sup> Jour. Infect. Dis., 1911, 9, p. 366.

With liver extracts 32% of the tests were positive, with kidney extracts 57%, and with lymph-gland extracts 68%. They consider that they have demonstrated the specific virus of scarlet fever as being present especially in the lymphatic glands. Isabolinsky and Legeika<sup>16</sup> used both watery and alcoholic extracts of scarlatinal liver, spleen, and lymph glands. Unlike the Koesslers, they obtained positive reaction in both scarlatinal and control sera and conclude that the fixation obtained is not specific.

TABLE 1

THE PERCENTAGE OF CASES OF SCARLET FEVER GIVING POSITIVE WASSERMANN TESTS ACCORDING TO VARIOUS OBSERVERS

Observer	Kind of Antigen Employed	Percentage Positive
Much and Eichelberg <sup>1</sup> .....	Watery extract of syphilitic liver.....	40
Teissier and Bernard <sup>2</sup> .....	Watery extract of syphilitic liver.....	84
Haendel and Schultz <sup>3</sup> .....	Watery extract of syphilitic liver.....	13
Jochmann and Toepfer <sup>4</sup> .....	Watery extract of syphilitic liver.....	3
Jacobovics <sup>5</sup> .....	Not stated.....	33
Zeissler <sup>6</sup> .....	Alcoholic extract of human heart.....	4.8
Bruck and Cohn <sup>7</sup> .....	Alcoholic extract of syphilitic liver.....	2.8
Boas and Hange <sup>8</sup> .....	Alcoholic extract of human heart.....	1.6
Hecht, Latelner and Wilenko <sup>9</sup> .....	Alcoholic extract of guinea-pig heart.....	1
Meier <sup>10</sup> .....	Watery extract of syphilitic liver.....	0
Schleissner <sup>11</sup> .....	Alcoholic extract of guinea-pig heart.....	0
Seligman and Klopstock <sup>12</sup> .....	Alcoholic extract of human heart:	
	1st series.....	0
	2nd series deteriorated antigen.....	100
Halberstaedter, Müller, and Reiche <sup>13</sup> ..	Alcoholic extract of syphilitic liver:	
	One antigen.....	50
	All other antigens.....	0
*Kolmer <sup>14</sup> .....	Alcoholic extract of syphilitic liver.....	2
Gross and Folk <sup>15</sup> .....	Alcoholic extract of human heart.....	0
Hoehne <sup>16</sup> .....	Alcoholic extract of syphilitic liver.....	0
Sommerfeld <sup>17</sup> .....	.....	0
Noguchi <sup>18</sup> .....	Acetone-insoluble extract of beef heart.....	0
*Author's observations.....	Acetone-insoluble extract of beef heart controlled in 6 cases by cholesterinized alcoholic extract of human heart.....	1
Fua and Koch <sup>19</sup> .....	.....	0

\* Syphilis not excluded.

<sup>1</sup> Med. Klin., 1908, 4, p. 671.

<sup>2</sup> Compt. rend. Soc. de biol., 1910, 68, p. 272.

<sup>3</sup> Ztschr. f. Immunitätsf., 1908-9, 1, p. 91.

<sup>4</sup> München. med. Wehnschr., 1908, 4, p. 1690.

<sup>5</sup> Jahrb. f. Kinderh., 1914, 29, p. 215.

<sup>6</sup> Berl. klin. Wehnschr., 1908, 14, p. 1887.

<sup>7</sup> Ibid., p. 2268.

<sup>8</sup> Ibid., p. 1566.

<sup>9</sup> Ztschr. f. Immunitätsf., 1909, 2, p. 356.

<sup>10</sup> Med. Klin., 1908, 4, p. 1333.

<sup>11</sup> Wien. klin. Wehnschr., 1908, 21, p. 1375.

<sup>12</sup> Berl. klin. Wehnschr., 1908, 45, p. 1710.

<sup>13</sup> Ibid., p. 1917.

<sup>14</sup> Jour. Exper. Med., 1911, 14, p. 235.

<sup>15</sup> Wien. klin. Wehnschr., 1908, 21, p. 1522.

<sup>16</sup> Berl. klin. Wehnschr., 1908, 14, p. 1717.

<sup>17</sup> Arch. f. Kinderh., 1909, 1, p. 38.

<sup>18</sup> Serum Diagnosis of Syphilis, 1910.

<sup>19</sup> Wien. klin. Wehnschr., 1909, 22, p. 522.

In interpreting these varying results much valuable information may be obtained by a review of the Wassermann test in scarlet fever. Much and Eichelberg,<sup>17</sup> using watery extract of syphilitic liver as antigen, obtained positive results in 40% of scarlet-fever cases, and they concluded that the Wassermann reaction was not specific for syphilis.

<sup>16</sup> Centralbl. f. Bacteriol., I, O., 1913, 71, p. 520.

<sup>17</sup> Med. Klin., 1908, 6, p. 671.

Following this work, which unfortunately has given rise to the idea that the Wassermann test is commonly positive in scarlet fever, a few other reports appeared giving positive results. These reports are summarized in Table 1. It will be seen that the watery extracts of organs gave the highest percentage of positive tests as well as the greatest variation in the hands of different observers. The alcoholic and acetone-insoluble extracts, except when deteriorated, give practically uniformly negative results in scarlet-fever patients in whom syphilis could be excluded, both according to others' results and according to our own results in 100 cases.

The work of Seligman and Klopstock and Halberstaedter illustrates what variation in results can be obtained with a change in the antigen employed. The watery extracts used as antigen gave such a high percentage of positive results with normal and other sera that they have been abandoned as antigens for the Wassermann test for syphilis. From the foregoing considerations it is plain that positive results in fixation tests in which watery extracts of organs serve as antigen, must be interpreted with great reserve. Recently Schultz<sup>18</sup> used washed scarlatinal lymph glands as antigens for Abderhalden reactions in scarlet fever. He obtained positive results with the sera of scarlatina patients, but he also obtained positive results when he used normal lymph glands as antigen. He concludes that the reaction is not connected with any specific virus for scarlet fever.

#### THROAT AND MOUTH SECRETIONS

Though not proved, it is commonly assumed that the angina associated with scarlet fever is the source of a general intoxication due at least to secondary infection if not to the specific virus of the disease. Mucus from the throat and mouth of cases with active angina was collected and used as antigen for complement-fixation with a method such as described in the foregoing tests.

The mucus from cases with active angina was suspended in salt solution and by preliminary titration a concentration obtained which made convenient quantities. The following quantities were used: 0.01 c.c., 0.02 c.c., 0.03 c.c., 0.05 c.c., 0.1 c.c., 0.15 c.c., 0.2 c.c., 0.3 c.c., and 0.5 c.c. The mucus was used fresh in all but one instance; here it was used both fresh and heated to 60 C. for 1 hour. The serum was employed in constant quantity, 0.1 c.c., and inactivated except in one test, in which it was used in both active and inactive form. For each test a control with serum from diphtheria patients was made. As in all the experiments, Wassermann tests were made with serum from scarlet fever. In one case no inhibition was obtained by means of antigen alone in any of the quantities used. In the other tests the antigen produced partial inhibition in the highest quantities.

<sup>18</sup> München. med. Wchnschr., 1913, 60, p. 2510.

The results of the tests were as follows: In sera from 4 cases of scarlet fever in various stages of convalescence, each used with a different antigen, there was no evidence of complement-fixation. As a rule the diphtheria serum inhibited hemolysis with lower strengths of antigen than did the scarlatinal serum. In a 5th case there was a slight fixation of complement with 0.3 c.c. of antigen. There was no fixation of complement at all in the control up to 0.5 c.c. The same antigen failed to fix complement with the serum of a 6th case of scarlet fever.

#### SUMMARY

Complement-deviation and cutaneous tests failed to demonstrate any specific scarlatinal virus or antigen in the blood serum or in extracts of spleen or lymph glands.

Fixation tests with throat mucus as antigen gave in one instance a weak fixation of complement with the serum of a convalescent scarlatinal patient.

On the basis that insufficient concentration of antigen explains the negative results, it would appear desirable to study further the cultures obtainable from scarlet fever, particularly as to their immune reactions.

The toxicity of human blood serum for guinea-pigs is increased during the acute stage of scarlatina.

Positive Wassermann reactions in scarlet fever obtained with the acetone-insoluble portion of alcoholic heart extract are strongly suggestive of syphilis.

# ANAPHYLAXIS REACTIONS BETWEEN PROTEINS FROM SEEDS OF DIFFERENT GENERA OF PLANTS \*

## THE BIOLOGIC REACTIONS OF THE VEGETABLE PROTEINS, VII

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Recent investigations have clearly shown that the specificity of the anaphylaxis reaction depends upon the chemical constitution of the protein, and not upon its biologic origin. The biologic specificity shown when animal fluids or vegetable extracts are used as antigens is consequently due to the fact that the protein constituents of these fluids, or extracts, are specific for each organism. The specificity of the anaphylaxis reaction is therefore an expression of the specificity of the proteins for the plant or animal from which the material for the experiments was derived. Whether this specificity which is revealed by the anaphylactic reaction implies identity of chemical constitution, or the presence of identical groups, or radicals, in the molecules of the proteins inducing this reaction, cannot be definitely settled until some method is discovered by which the chemical individuality of protein preparations can be positively demonstrated.

In numerous instances we have found that carefully purified preparations of a protein from one species can sensitize guinea-pigs to equally carefully purified preparations of physically and chemically similar, tho apparently structurally different, proteins from another species. Thus we have shown that guinea-pigs sensitized with gliadin from wheat are intoxicated with hordein from barley, the reaction being almost as strong as when they are intoxicated with gliadin from wheat. The same is true when the order of treatment is reversed.<sup>1</sup> Legumin from the pea renders guinea-pigs as sensitive to legumin from the vetch as it does to itself and vice versa. In other words these proteins, from seeds of different genera, behave anaphylactically as tho they were identical proteins.

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<sup>1</sup> Wells and Osborne: Jour. Infect. Dis., 1913, 12, p. 341.

In this paper we propose to discuss further the reactions obtained between proteins of similar chemical properties obtained from seeds of different genera. The results of experiments already published in our earlier papers are included in this discussion together with new experiments which have been made in order to obtain further data on this subject.

# PROTEINS FROM LEGUMINOUS SEEDS

## LEGUMIN

This, the principal protein obtained from the seeds of the pea (*Pisum sativum*), the vetch (*Vicia sativa*), the lentil (*Ervum lens*), and the horse bean (*Vicia faba*), has been the subject of much study. A comparison of carefully purified preparations from each of these four seeds has failed to reveal any differences, discoverable by chemical means, which are sufficient to show that they are not identical in constitution.<sup>2</sup>

TABLE 1  
LEGUMIN

Substance	No Reaction	Doubtful	Slight	Moderate	Severe	Fatal	Protection
Pea + pea.....	2	1	1	6	2	0	
Vetch + vetch.....	2	2	3	5	1	2	
Lentil + lentil.....	0	0	0	0	2	0	
Horse bean + horse bean..	0	0	0	0	1	1	
Pea + vetch.....	0	2	0	3	1	0	Incomplete
Pea + lentil.....	0	0	0	0	2	0	Complete?
Pea + horse bean.....	0	0	0	3	0	0	Complete
Vetch + pea.....	0	4	3	3	0	0	Partial
Vetch + lentil.....	0	0	0	0	0	2	No experiments
Vetch + horse bean.....	0	0	0	0	2	0	Complete
Lentil + pea.....	0	0	0	2	0	0	None
Lentil + vetch.....	0	0	0	1	1	0	None
Lentil + horse bean.....	0	0	0	1	1	0	Complete
Horse bean + pea.....	0	0	3	0	2	0	Partial
Horse bean + vetch.....	0	0	0	0	0	2	No experiments
Horse bean + lentil.....	0	0	0	0	1	1	Complete

Table 1 gives the results of anaphylaxis experiments which were made with pure preparations of legumin from each of these seeds. The methods of conducting these experiments and describing the results are the same as given in our first paper of this series.<sup>3</sup> The numbers in the table show the number of experiments that were tried, with the outcome indicated at the head of the column. The sensitizing dose was

<sup>2</sup> Osborne and Campbell: Jour. Am. Chem. Soc., 1898, 20, p. 410. Osborne and Heyl: Am. Jour. Physiol., 1908, 22, p. 423, and Jour. Biol. Chem., 1908, 5, p. 187.

<sup>3</sup> Wells and Osborne: Jour. Infect. Dis., 1911, 8, p. 66.

from 1 to 5 mg., the incubation period from 16 to 21 days, and the intoxicating dose from 50 to 100 mg. Animals recovering from the intoxicating dose were given a second dose of the same protein 24 hours later to assure saturation with this protein. After a further interval of from 48 to 72 hours they received from 50 to 100 mg. of the protein used for sensitizing to determine whether or not they had been protected by the heterologous protein.

These results show that preparations of legumin from each of these four seeds react with one another as tho they were homologous proteins, the number of severe or fatal reactions between the preparations of different origin being as great as when preparations from the same seed are used. While in most cases the guinea-pig was rendered refractory by the heterologous preparation, there were several cases in which no protection was afforded. In all the latter, except the reaction between legumin from the lentil and the vetch, the legumin from the pea was involved. The data given in the table indicate that the anaphylactogenic power of the pea legumin was somewhat less than that of the preparations from the other three seeds. A possible explanation may ultimately be found in the fact that the preparation of pea legumin used for these experiments had been subjected to repeated fractional precipitation from ammonium sulfate solution, whereas the other preparations had been purified by reprecipitation from sodium-chlorid solutions. There is no apparent reason why this difference in the method of purification should affect the result of anaphylaxis experiments.

These experiments are in harmony with the conclusion drawn from chemical comparisons that preparations of legumin from each of these seeds are, if not chemically identical, at least extremely similar to one another in their chemical constitution.

#### VICILIN

The seeds of the pea, lentil, and horse bean contain besides legumin a smaller amount of another protein known as vicilin. This differs from legumin in being coagulable by heat, and more soluble in very dilute salt solutions. It also has a slightly different ultimate composition and yields somewhat different proportions of some of the products of acid hydrolysis. It is very difficult to separate vicilin from legumin and furthermore there is no way in which to show that preparations of vicilin contain no legumin. Since seeds of the vetch yield no vicilin, animals sensitized with vetch legumin should not react when intoxicated with pea vicilin unless these heterologous proteins are capable of

reacting anaphylactically with one another. In the following table this is shown to be the case, pronounced reactions being obtained in nearly all of the experiments, altho the animals were not made refractory. When these experiments were tried with vicilin and pea legumin the reactions were far more severe than when the guinea-pigs were sensitized and intoxicated with pea legumin alone, possibly because vicilin is much more soluble in very dilute salt solutions than is legumin. Table 2 gives the results of these experiments.

TABLE 2  
VICILIN

	No Reaction	Doubtful	Slight	Moderate	Severe	Fatal	Protection
Vicilin, pea + vicilin, pea..	0	0	2	1	4	2	
Legumin, vetch + vicilin, pea.....	0	0	0	5	1	3	None
Vicilin, pea + legumin, vetch.....	0	1	2	0	4	0	None
Legumin, pea + vicilin, pea.....	0	0	0	0	0	4	No experiments
Vicilin, pea + legumin, pea.....	0	0	0	0	1	3	No experiments

## VIGNIN

The seeds of the cow-pea (*Vigna catjang*) contain a large proportion of a protein named vignin, which resembles legumin in chemical and physical properties, but differs sufficiently to leave no doubt that it is a distinctly different protein. The reactions given in the following table indicate that vignin may react with the similar heterologous proteins, legumin from the vetch, vicilin, and glycinin (from the soy bean), but the results of these experiments are irregular and cannot be explained by any of the facts known concerning these proteins. Only in the crossed reaction with vetch legumin are the reactions usually strong enough to be a satisfactory indication of relationship, while only with pea legumin can relationship be excluded by absence of reaction.

TABLE 3  
VIGNIN

	No Reaction	Doubtful	Slight	Moderate	Severe	Fatal	Protection
Vignin + vignin.....	0	0	0	0	2	4	
Vignin + legumin, pea.....	0	4	0	0	0	0	None
Legumin, pea + vignin.....	0	8	0	0	0	0	None
Vignin + legumin, vetch....	2	2	0	1	2	2	None or part
Legumin, vetch + vignin....	4	0	1	1	2	0	None
Vignin + vicilin, pea.....	0	0	4	4	0	0	?
Vicilin, pea + vignin.....	3	1	2	0	0	0	None
Vignin + glycinin.....	3	1	0	0	0	0	None
Glycinin + vignin.....	1	0	2	2	3	0	None

## GLYCININ

The greater part of the protein of the soy bean (*Soja hispida*) has been named glycinin. It resembles legumin, vicilin, and vigin in most of its chemical and physical properties, but is not identical with any one of these proteins.

While guinea-pigs both sensitized and intoxicated with glycinin react severely, or fatally, there was no reaction when the experiments were made between glycinin and vetch legumin, or pea vicilin, sufficiently pronounced to indicate that these proteins react anaphylactically with one another.

Five of 8 animals sensitized with glycinin gave pronounced reactions when subsequently injected with vigin and 2 of the remaining 3 reacted slightly. There was no protection observed in the animals thus treated. The results of the reactions given in Table 4 show no reaction between glycinin and vicilin from the pea, or legumin from the vetch.

TABLE 4  
GLYCININ

	No Reaction	Doubtful	Slight	Moderate	Severe	Fatal	Protection
Glycinin + glycinin.....	0	0	0	1	3	3	
Glycinin + vigin.....	1	0	2	2	3	0	None?
Vigin + glycinin.....	3	1	0	0	0	0	None
Glycinin + vicilin, pea.....	0	3	0	1	0	0	None
Vicilin, pea + glycinin.....	0	0	3	0	0	0	
Glycinin + legumin, vetch...	2	0	2	0	0	0	Partial
Legumin, vetch + glycinin..	5	0	0	0	0	0	None

## PHASEOLIN

The kidney bean (*Phaseolus vulgaris*) yields a relatively large amount of protein soluble in dilute salt solutions, which has been named phaseolin. The Japanese adzuki bean (*Phaseolus radiatus*) likewise yields a protein so similar to that obtained from the kidney bean that a very careful comparison of purified preparations from these two sources has shown no differences in chemical and physical properties except a slightly larger proportion of basic nitrogen in those from the adzuki bean.<sup>4</sup>

As shown in Table 5, preparations from these two seeds did not react anaphylactically with one another and are consequently to be regarded as distinctly different proteins. It is striking that no reaction

<sup>4</sup> For the properties of these two proteins cf. Osborne, Jour. Am. Chem. Soc., 1894, 16, pp. 633, 703, 757; also Osborne and Campbell, *ibid.*, 1897, 19, p. 509; also Osborne and Harris, *ibid.*, 1903, 25, p. 336.

whatever should have been obtained between proteins apparently so similar and derived from seeds of plants so nearly alike that they have been assigned to the same genus.

TABLE 5  
PHASEOLIN

	No Reaction	Doubtful	Slight	Moderate	Severe	Fatal	Protection
Phaseolin, adzuki bean + phaseolin, adzuki bean....	0	0	0	0	0	1	
Phaseolin, adzuki bean + phaseolin, kidney bean....	5	1	0	0	0	0	None
Phaseolin, kidney bean + phaseolin, kidney bean....	0	0	0	0	0	1	
Phaseolin, kidney bean + phaseolin, adzuki bean....	5	1	0	0	0	0	None

### PROTEINS FROM SEEDS OF CEREALS

GLIADIN AND GLUTENIN FROM WHEAT, GLIADIN FROM RYE, HORDEIN FROM BARLEY, AND THE ALCOHOL-SOLUBLE PROTEINS FROM OATS AND SORGHUM

The seeds of wheat (*Triticum vulgare*) and rye (*Secale cereale*) contain proteins soluble in 70% alcohol which are so nearly alike in chemical and physical properties that the only positive difference between the preparations from these two seeds that has as yet been established is a somewhat higher specific rotation for those from rye. From this it is probable that the alcohol-soluble protein from wheat, gliadin, is not identical with that from rye.

The seeds of wheat also contain a considerable amount of another protein, glutenin, which is not soluble in 70% alcohol and in several other respects differs so much from gliadin as to leave no doubt that these two proteins are distinctly different.

The seeds of barley (*Hordeum vulgare*) contain a considerable amount of protein soluble in alcohol, hordein, which, tho resembling gliadin, has been shown to be distinctly different. The anaphylactic relations of these proteins have been so extensively discussed in the second paper of this series<sup>5</sup> that it is not necessary to comment on them further. In that paper we stated that "we must conclude from the results of our experiments either that our preparations of gliadin and hordein each contain two different proteins, one of which is common to both preparations, or that they contain at least two reactive groups, one of which is common to both proteins, each of which groups behaves as a distinct antigen when injected into guinea-pigs."

<sup>5</sup> Wells and Osborne: Jour. Infect. Dis., 1913, 12, p. 341.

Groh and Friedl<sup>6</sup> have recently concluded from a comparison of the physical properties of successive fractional precipitates of carefully purified gliadin that this consists of a single protein; hence it is now highly probable that the anaphylactic reactions between gliadin and hordein are due to the presence of common reactive groups in these chemically different proteins.

Preparations of protein soluble in relatively strong alcohol have been made from seeds of the oat<sup>7</sup> (*Avena sativa*) and also from those of the sorghum.<sup>8</sup> Neither of these proteins has been studied in detail and practically nothing is known about their chemical characteristics. Some anaphylaxis experiments have been made with crude preparations of the two latter proteins.

Table 6 summarizes the results of our experiments with these six proteins from seeds of different genera.

TABLE 6

SUMMARY OF THE RESULTS OF EXPERIMENTS WITH PROTEINS FROM SEEDS OF DIFFERENT GENERA

	No Reaction	Doubtful	Slight	Moderate	Severe	Fatal	Protection
Gliadin, wheat + gliadin, rye..	0	0	0	1	3	4	Incomplete
Gliadin, rye + gliadin, wheat..	1	0	0	0	4	2	?
Gliadin, wheat + glutenin, wheat.....	0	0	0	6	9	1	Incomplete
Glutenin, wheat + gliadin, wheat.....	0	0	0	0	7	1	Complete?
Gliadin, rye + glutenin, wheat	0	0	2	4	2	0	None?
Glutenin, wheat + gliadin, rye.....	4	0	4	1	0	1	Complete?
Gliadin, wheat + hordein, barley.....	0	1	0	1	8	1	None
Hordein, barley + gliadin, wheat.....	0	0	0	0	9	3	None?
Gliadin, rye + hordein, barley	0	0	0	0	4	0	None
Hordein, barley + gliadin, rye.....	0	0	0	0	3	1	None
Alcohol-soluble protein, oat + alcohol-soluble protein, oat.....	0	0	2	0	0	0	
Alcohol-soluble protein, oat + zein, maize.....	2	0	0	0	0	0	
Alcohol-soluble protein, oat + gliadin, wheat.....	2	0	0	0	0	0	
Alcohol-soluble protein, oat + hordein, barley.....	2	0	0	0	0	0	
Alcohol-soluble protein, oat + alcohol-soluble protein, sorghum.....	2	0	0	0	0	0	
Alcohol-soluble protein, sorghum + alcohol-soluble protein, sorghum.....	0	0	0	0	1	1	
Alcohol-soluble protein, sorghum + gliadin, wheat.....	2	0	0	0	0	0	None
Alcohol-soluble protein, sorghum + hordein, barley.....	2	0	0	0	0	0	None
Alcohol-soluble protein, sorghum + alcohol-soluble protein, oat.....	2	0	0	0	0	0	None

<sup>6</sup> Biochem. Ztschr., 1914, 66, p. 154.<sup>7</sup> Osborne: Am. Chem. Jour., 1891, 13, pp. 327, 385; 1892, 14, p. 212.<sup>8</sup> Not published.

## JUGLANSIN AND CORYLIN

The American black walnut (*Juglans nigra*) and the hazel-nut (*Corylus avellana*) contain relatively large proportions of protein having the properties of globulin. They are very similar to one another in physical and chemical properties.<sup>9</sup> A careful comparison of these globulins, which have been named juglansin and corylin respectively, has revealed slight differences, which appeared to justify applying different names to them.<sup>10</sup> To determine whether or not this distinction was warranted we have tried the following experiments.

TABLE 7  
RESULTS OF EXPERIMENTS WITH JUGLANSIN AND CORYLIN

	No Reaction	Doubtful	Slight	Moderate	Severe	Fatal	Protection
Juglansin + juglansin.....	0	0	0	0	0	2	None
Corylin + juglansin.....	4	0	0	0	0	0	
Corylin + corylin.....	0	0	0	0	0	1	
Juglansin + corylin.....	2	3	0	0	0	0	None

The fact that these proteins have high anaphylactic power, yet do not react when one is used for sensitizing and the other for intoxicating, nor develop any protection, may be taken as positive evidence that they are chemically different substances.

In Table 8 the results of a number of experiments are given in which proteins were used which presumably are distinctly different from one another, yet might possibly contain common anaphylactically reactive groups. The outcome of these experiments shows that these proteins have nothing in common, in so far as the anaphylaxis reaction is concerned.

TABLE 8  
RESULTS OF EXPERIMENTS TO DETERMINE COMMON ANAPHYLACTICALLY REACTIVE GROUPS IN CORYLIN, EXCELSIN, AMANDIN, JUGLANSIN, AND HAZEL-NUT PROTEOSE

	No Reaction	Doubtful	Slight	Moderate	Severe	Fatal	Protection
Corylin + excelsin.....	2	0	0	0	0	0	None
Excelsin + corylin.....	2	0	0	0	0	0	None
Corylin + amandin.....	2	0	0	0	0	0	None
Amandin + corylin.....	2	0	0	0	0	0	None
Amandin + juglansin.....	2	0	0	0	0	0	None
Juglansin + amandin.....	2	0	0	0	0	0	None
Excelsin + juglansin.....	2	0	0	0	0	0	None
Juglansin + hazel-nut proteose.....	2	0	0	0	0	0	None

<sup>9</sup> Osborne and Campbell: Jour. Am. Chem. Soc., 1896, 18, p. 609.

<sup>10</sup> Osborne and Harris: Ibid., 1903, 25, p. 848.

## GLOBULIN FROM FLAX SEED, AND EDESTIN FROM HEMP SEED

The flax seed (*Linum usitatissimum*) and the hemp seed (*Cannabis sativa*), contain relatively large quantities of protein soluble in salt solutions and having properties characteristic of globulins. These crystallize in octahedra and closely resemble one another in physical properties and in the proportion of the decomposition products which they yield on hydrolysis with acids. Distinct differences have, however, been detected between them, so that there can be no doubt that they are not identical.

The immunologic relationships between edestin and flax-seed globulin are unusual. White and Avery<sup>11</sup> sensitized two guinea-pigs with edestin, which reacted fatally when flax-seed globulin was injected intravenously (using 40 and 120 times the fatal dose of edestin). Lake<sup>12</sup> found (Experiment 11) that an antiserum for edestin which gave a positive complement-fixation reaction with edestin at 1:50,000, and a precipitin reaction at a dilution of 1:100,000, when injected into guinea-pigs, made them strongly sensitive (passive anaphylaxis) to

TABLE 9  
EXPERIMENTS IN ANAPHYLAXIS

Sensitizing Dose	Second Dose	Route*	Result	Protection Test Reaction
1. Flax seed globulin	Edestin.....	Peritoneum	0	Moderate
2. Flax seed globulin	Edestin.....	Peritoneum	0	Moderate
3. Flax seed globulin	Edestin.....	Peritoneum	Slight	Moderate
4. Flax seed globulin	Edestin.....	Peritoneum	Slight	Severe
5. Flax seed globulin	Edestin.....	Heart	0	
6. Flax seed globulin	Edestin.....	Carotid	Died, 2 min.	
7. Flax seed globulin	Edestin.....	Heart	Slight	Severe
8. Flax seed globulin	Edestin.....	Heart	0	Severe
9. Flax seed globulin	Edestin.....	Heart	Slight	Severe
10. Flax seed globulin	Edestin.....	Heart	Died, 90 sec.	
11. Flax seed globulin	Edestin.....	Vein	Moderate	Moderate
12. Flax seed globulin	Edestin.....	Heart	0	Died, 2 min., heart
13. Flax seed globulin	Edestin.....	Heart	0	
14. Flax seed globulin	Edestin.....	Carotid	0	
15. Edestin.....	Flax seed globulin	Peritoneum	0	Moderate
16. Edestin.....	Flax seed globulin	Peritoneum	0	Moderate
17. Edestin.....	Flax seed globulin	Peritoneum	0	Moderate
18. Edestin.....	Flax seed globulin	Peritoneum	Doubtful	Moderate
19. Edestin.....	Flax seed globulin	Heart	Doubtful	Moderate
20. Edestin.....	Flax seed globulin	Heart	Doubtful	Moderate
21. Edestin.....	Flax seed globulin	Heart	Slight	Moderate
22. Edestin.....	Flax seed globulin	Heart	Died, 4 min.	
23. Edestin.....	Flax seed globulin	Heart	Moderate	Slight
24. Edestin.....	Flax seed globulin	Heart	0	Died, 20 min., heart
25. Edestin.....	Flax seed globulin	Carotid	0	
26. Edestin.....	Flax seed globulin	Jugular	0	

\* In the case of intravascular injections the dose was but 5 mg.

<sup>11</sup> Jour. Infect. Dis., 1913, 13, p. 103.

<sup>12</sup> Lake, Osborne, and Wells: Ibid., 1914, 14, p. 364.

edestin. This antiserum also gave a precipitin reaction with flax-seed globulin at 1:10,000 and complement-fixation at the same dilution. Evidently some relation exists here. We have performed anaphylaxis experiments as indicated in Table 9.

With these proteins we find that usually no crossed reactions are obtained, but in 18 experiments in which the heterologous protein was introduced directly into the blood of the sensitized animal, 3 fatal reactions, characteristic of anaphylaxis, were obtained. We are not prepared to explain these exceptional results.

#### COMPARISON OF CHEMICALLY DISTINCT PROTEINS OF THE SAME SEEDS

The data thus far discussed show that the typical and severe anaphylaxis reactions may sometimes be obtained when proteins isolated from seeds of different genera are employed for the sensitizing and intoxicating doses in the same animal. In nearly every case such reactions have been developed only by preparations of proteins so nearly alike that differences between them have not been detected by physical or chemical means, or the differences found have been so slight that it seems highly probable that the proteins concerned are very similar in chemical constitution.

The only cases in which positive reactions have been obtained between proteins which chemical tests have indicated to have distinct differences in their constitution are vicilin (pea) versus legumin (vetch); vigin (cow pea) versus legumin (vetch); hordein (barley) versus gliadin (wheat or rye); and gliadin (wheat) versus glutenin (wheat). Such reactions can be attributed to the existence of common reactive groups in these different proteins, evidence of which we have given in the second paper of this series.<sup>13</sup>

In the first paper<sup>14</sup> of this series a list of reactions was given on page 119 between proteins of different origin and apparently different constitution in which the outcome was such that these reactions were designated doubtful. Further investigations must be made before these irregular results can be explained.

Table 10 gives the results of experiments which we have made with preparations derived from the same seed but of doubtless different chemical constitution.

The slight reactions caused by several of the preparations from the same seed are in marked contrast to the severe reactions obtained when the homologous proteins were used, and may consequently be

<sup>13</sup> Wells and Osborne: *Jour. Infect. Dis.*, 1913, 12, p. 341.

<sup>14</sup> Wells and Osborne: *Ibid.*, 1911, 8, p. 66.

TABLE 10

RESULTS OF EXPERIMENTS MADE WITH PROTEINS FROM THE SAME SEED BUT OF DIFFERENT CHEMICAL CONSTITUTION

	No Reaction	Doubtful	Slight	Moderate	Severe	Fatal	Protection
Globulin, castor bean + protease.....	0	0	2	0	0	0	None
Protease, castor bean + globulin.....	1	2	0	0	0	0	None
Flax seed protease + globulin.....	0	0	1	1	0	0	Partial
Flax seed globulin + protease.....	0	0	1	1	0	1	None
Edestin + hemp seed protease.....	0	0	2	0	0	0	None
Hemp seed protease + edestin.....	2	0	3	0	1	0	None
Protease, pea + legumin, pea.....	0	0	2	0	0	0	None
Protoprotease, pea + legumin, pea.....	0	1	0	0	0	0	Partial
Vicilin, pea + protease, pea.....	1	1	0	0	0	0	Partial
Vicilin, pea + Protoprotease, pea.....	0	0	1	0	0	0	Partial
Legumin, pea + protease, pea.....	1	1	0	0	0	0	Partial
Legumin, pea + Protoprotease pea.....	0	0	1	1	0	0	Partial
Protease, lentil + legumin, lentil.....	4	0	0	0	0	0	None
Legumin, lentil + protease, lentil.....	2	0	0	0	0	0	None
Protease, soy bean + glycinin.....	0	0	2	0	0	0	Partial
Glycinin, soy bean + protease.....	0	0	0	1	1	0	Partial
Globulin, adzuki bean + protease.....	0	0	1	1	0	1	Partial
Protease, adzuki bean + globulin.....	2	0	1	0	0	0	Partial

ascribed to an incomplete separation of the proteins. That this is highly probable is indicated by the few cases where more severe reactions are recorded, for such results occurred when the globulin was used for sensitizing and the protease for intoxicating. Since the reversed reactions were much less severe and since the globulins, owing to the methods employed in their preparation, might easily contain traces of protease, it is much more probable that these reactions were caused by incomplete separation of the two proteins than by a reaction between them.

## CONCLUSION

Since chemically similar proteins from seeds of different genera react anaphylactically with one another, while chemically dissimilar proteins from the same seed in many cases fail to do so, we must conclude that the specificity of the anaphylaxis reaction depends upon the chemical structure of the protein molecule.

# CASES OF GENERALIZED FATAL BLASTOMYCOSIS, INCLUDING ONE IN A DOG \*

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A number of cases of generalized fatal blastomycosis have been reported previously by Hektoen, Ricketts, Bassoe, Gilchrist, Hyde, Montgomery, Curtis, and others. We wish to add to the list of cases already published two more of the same kind occurring in man, while Dr. Maximilian Herzög furnishes a brief statement of a case of generalized fatal blastomycosis in a dog, apparently the only one in this species of domestic animal ever observed. Blastomycosis in the horse has been observed in various countries a number of times, tho it is now claimed that most of what heretofore has been described as equine blastomycosis is really a sporotrichosis.

The micro-organisms from the human cases reported in this paper, have been examined fully by cultural methods and also by animal experiments. The blastomyces obtained in pure cultures from two generalized fatal cases and from a cutaneous case, did not appear to be very virulently pathogenic for the animals used in the experiments, tho lasting pathologic lesions were produced in some animals, as will appear from the detailed report which follows.

## CASE 1

The patient was an Italian street laborer, 30 years old, married, with 2 healthy children. His parents were living and in good health; his 4 brothers and 2 sisters were also all living and well. Patient denied syphilis or any severe previous illness. Usual weight 140 pounds. While working as an excavator for a gas main, October 4, 1913, he was troubled with a cough which was diagnosed as bronchitis. There was considerable expectoration, but no tubercle bacilli were found. The patient complained of dizziness. A warty growth of the upper lip was first noticed by the patient the latter part of December, but this was not accompanied by pain, soreness or itching; there were no other skin lesions at this time. Early in February, 8 abscesses began to form in various places over the body. The patient's temperature throughout ranged from 99 to 102 F., pulse from 90 to 100, respiration from 20 to 26, and there was up to February, 1914, a great loss of weight.

Treatment: The bronchitis was treated with the usual remedies, but with little improvement; as the abscesses developed, however, the cough practically disappeared. A number of the abscesses were lanced, and cleansed with tincture

\* Received for publication February 4, 1916.

of iodin; one was entirely healed, and the others were improved. The growth on the lip received applications of copper sulfate, which reduced the size of it somewhat. Internally potassium iodid was administered in large doses, also with copper sulfate in 1/6-gr. doses. April 11, 1914, the patient's condition was much improved, and there was decided gain in weight. The patient was seen again in May, after which he was lost track of, until his death. Postmortem examination was not permitted.

#### CASE 2

The patient was a woman 60 years old, an Italian, married (family history not obtainable). The woman had worked in a cellar sorting winter cabbages for many years. She had been troubled with a painful cough for over a year. After an accident in which she had fallen backward downstairs into the cellar, her arm became swollen and remained that way for 3 months. The cough was persistent until death. The body was literally covered with blastomycotic ulcers. No other history was obtainable and postmortem examination was not permitted.

#### CASE 3

The patient, an American machinist, aged 20, came to the clinic with a lesion on the ventral surface of the left wrist; no other complaint. Gonorrhea and syphilis denied, family history negative. The patient claimed that the lesion started as a pimple, which was accompanied at the onset with itching; it soon broke and discharged a glairy tenacious pus. The lesion, which kept getting larger, at the time of the examination was 3 by 1½ inches, with a slightly raised margin, and a dark varicose surface, scattered throughout which were little sinuses. Upon examination it was found that the patient was very sensitive to pain; on removal of the scab, a thick yellow discharge could be pressed out. Older areas of the ulcer had healed with cicatrix formation. Tubes were inoculated after the discharge had been examined in KOH solution, and after round doubly contoured bodies had been found. The patient had been working in a basement in Chicago for the past 6 months. On entering the clinic, he was given potassium-iodid treatment in large doses with the result that by November, 1914, the lesions had healed, leaving a dark disfiguring scar.

#### CULTURES

In Case 1, Dr. Maximilian Herzog and Dr. Margaret S. Grant obtained the blastomycotic organism from a blood culture, prepared in the usual manner. About 10 c.c. of the blood were drawn under all possible aseptic precautions from a vein of the patient's arm, and added at once to sterile broth in a flask and to agar tubes, which were then incubated. The cultures which developed first appeared, after from 3 to 4 days, as small colonies, granular and moist, dirty-white in color, and firmly adherent to the solid culture media. In the other two cases reported in this paper, the organisms were obtained from the cutaneous lesions.

The cultural characteristics of the organisms from the three cases studied were so similar that one common description for all may be given, with certain differential features, however, as will be pointed out.

*Agar Plates, Slants, and Stab Cultures.*—Aerobic growth; no growth anaerobically. On the agars—ascitic, glycerin, glucose, maltose, beer wort + 10 oxalic glucose + 10 oxalic lactose, and nutrient agar—the growth appeared on the late 3rd and early 4th days as little moist colonies, granular and pinpoint in size. very firmly attached to the media and difficult to remove, the colonies later



Fig. 1. Photograph of the face of Patient 2.



Fig. 2. Photograph of the arm of Patient 2, showing dermal lesions of blastomycosis.

becoming umbonate in shape. In from 7 to 10 days fine delicate aerial hyphae presented themselves. In from 12 to 15 days the colonies grew rapidly and presented a granular dirty-gray base supporting a fine raised white center from which, as mentioned in regard to colonies from Case 1, bush-like prominences (aerial hyphae) sprang (Fig. 3b). The surface now became uneven with an outgrowth of fine delicate closely woven threads, visible to the naked eye, which, being branched and at the edge of the growth attenuated, gave a ciliated appearance to the edge of the colony. In order to remove the colonies it was necessary to break the culture media with a strong platinum loop. The downy growth spread until after from 29 to 30 days it almost filled the tubes or covered the surface of the plates (Fig. 4), and generally showed small white spherical bodies throughout.

On glucose agar the growth was more rapid and the colonies became more coarsely granular and varicose than on the other media; microscopic examination showed the delicate threads that grew down into the media to be composed of segmented mycelia and round or oval doubly contoured buds. The segmented mycelia on all agars presented the same appearance, being from 4 to 6 to 10 microns in diameter, containing coarse and fine granules throughout the protoplasm. There were also seen clear, pink-colored, generally spherical vacuoles; nucleus-like structures were present in the segments; buds were seen in the more densely packed mycelial growth, which contained from 2 to 4 to 5 spherical bodies packed within the granular protoplasm. One budding process was seen coming off each segmented mycelial thread; this formation extended, became segmented, and formed new mycelial growths.

On glycerin agar the colonies as on other agars appeared in from 3 to 5 days, but did not present the coarsely granular appearance so pronounced on agar or glucose agar. The colonies on the 12th and 15th days showed slight cracks occurring in the surface, soon presenting the picture of a crumpled cloth (Fig. 6), all other characteristics remaining the same.

On ascitic agar aerial hyphae were less pronounced. Maltose-agar and beer-wort-agar growths showed the same characteristics as the growths on other agars. Nutrient agar growth was marked by fine granular moist pinpoint colonies that appeared on the 4th and 5th days. The growth was slower and poorer on this medium than on any of the other agars. Pieces of agar growth were used in 3 cases on Dogs 1 and 2 for inoculations.

*Broth.*—On + 10 acid, neutral, or — 10 alkaline broth growth occurred with no noticeable differences on the 4th and 5th days, but it was not so thick and luxuriant as on 2% glucose broth. Up to the 12th day of growth the colonies appeared as fine white fluffy balls, adherent to each other, and looking like thistle-down. No separation of these colonies occurred until after from 15 to 18 days' growth in all 3 cases. In cultures from Cases 2 and 3 after the 4th and 5th generation the colonies separated easily upon shaking. Top growth was not characteristic of any one of the three strains, but a bottom growth was typical; when, however, the growth had filled most of the liquid medium, i. e., in 5 or 6 weeks, a top growth also occurred. In cultures from Case 1, coarse spike-like aerial hyphae were present, springing from the top growth for 3 generations before the conditions ceased. The individual colonies were uniform in density, showing a dark-gray granular center, with fine closely woven offshoots which looked like projecting cilia at the edge of the floating colonies; 2 dark encircling rings showed in the older growths, due to the compact old growth of mycelial threads and the beginning of the younger straighter threads.



(a)

(b)

(c)

Fig. 3. (a) Pure culture, 6 months old, on glycerin agar, of the organism obtained from Patient 1. The culture was kept at room temperature. (b) Pure culture of the same organism after 7 days' growth on glucose agar in the incubator at 37 C. (c) Pure culture, 4 months old, on glycerin agar, kept at room temperature. Organism from Patient 2.

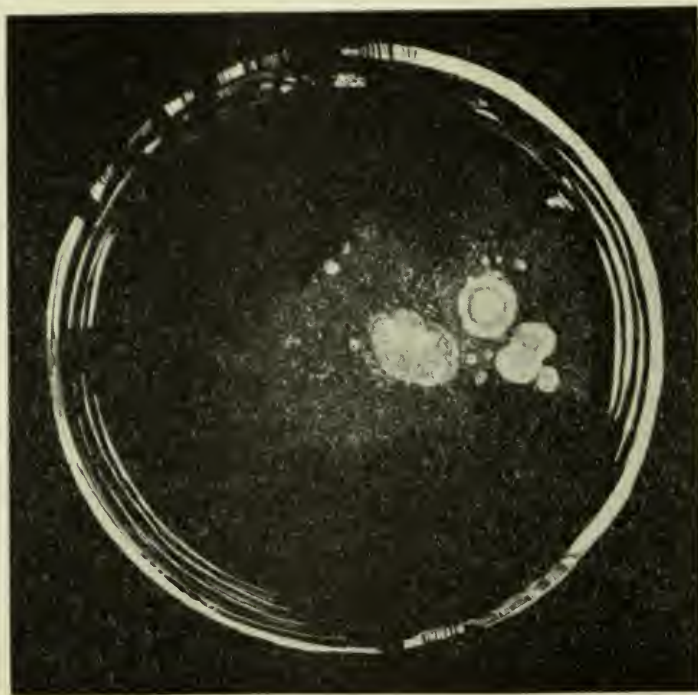


Fig. 4. Pure culture, 60 days old, on agar in a petri dish kept in the incubator at 37 C. Organism from Patient 2.

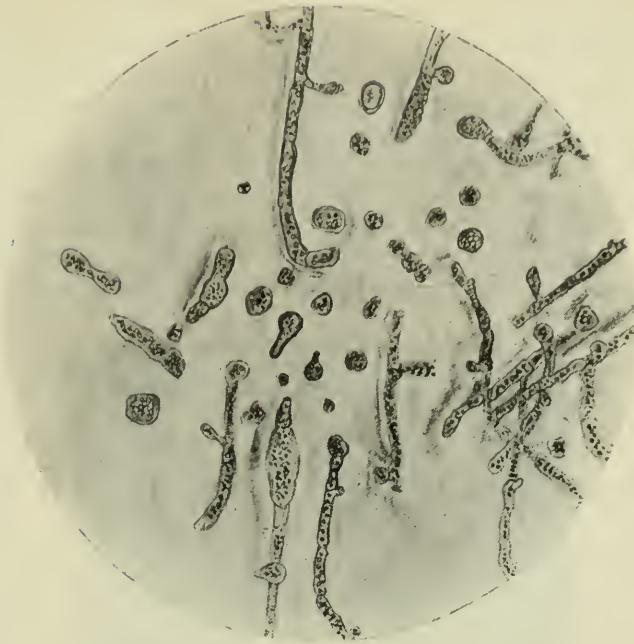


Fig. 5. Microscopic preparation of organism from Patient 2, from a young colony of a culture from 10 to 12 days old, showing formation of buds and mycelial threads. Spores in center of buds. Oil-immersion magnification.

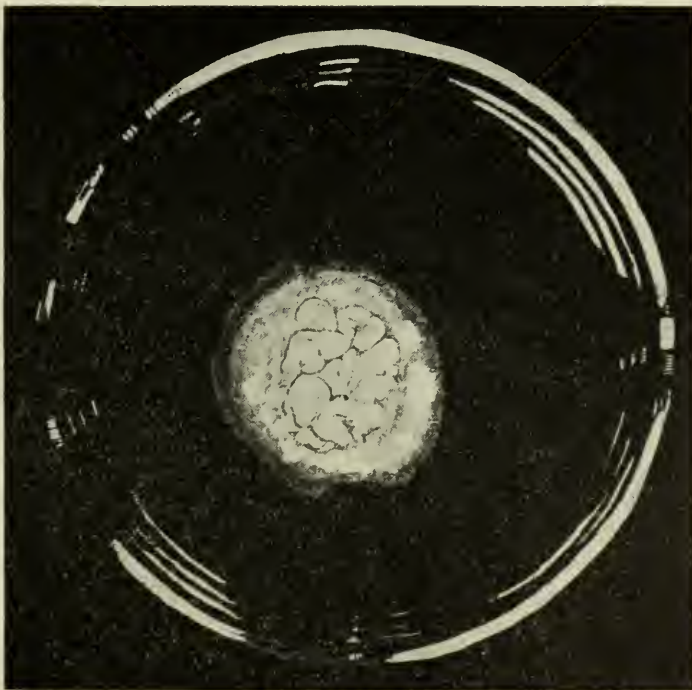


Fig. 6. Pure culture, 60 days old, on glycerin agar, kept at incubator temperature of 37 C. Organism from Patient 2.

Top growths soon became covered by a fine downy filamentous covering, dry and white in appearance. The growth was tenacious and resisted breaking up, but whole colonies could be completely removed with the platinum loop. Older colonies showed a degenerative change in the center, surrounded by gray granular spots (Fig. 7); growth consisted chiefly of mycelial threads with round or oval forms, scattered in the more tangled older threads. The microscopic findings were the same as on other media.

Broth cultures shaken with glass beads in physiologic salt solution were injected in 2- and 5-c.c. amounts, subcutaneously and intraperitoneally, into Guinea-pigs 1, 2, and 3, below, and behind the shoulder, and into Rabbits 1 and 2, and an atomizer was used to spray the animals with a view to producing the pulmonary form of blastomycosis if it could be produced. Rabbit 3 was injected



Fig. 7. Pure culture in nutrient broth, 40 days old, kept at room temperature. Organism from Patient 3.

intravenously. White Rats 1 and 2 received intravenous injections together with subcutaneous injections of 0.5 c.c. of bead-macerated broth. White Mice 1, 2, and 3, and Gray Rats 1 and 2 received the same.

*Koch's Blood Serum and Oxblood Serum.*—Growth occurred, as on other media, on the 3rd and 4th days as small gray pinpoint colonies, with fine ciliated edges growing out and into the media, the spike-like aerial hyphae being blunter and coarser, in cultures from Case 1, than on other media. All colonies grew rapidly and on the 10th day of growth the heavy wrinkled bases grew together, giving the surface a rugose or verrucose appearance. The colonies were so firmly incorporated into the media that removal necessitated a considerable amount of force, the growth coming away as a thick tenacious covering  $\frac{1}{32}$  inch in thickness. The fine downy mouldy growth was never as prominent on any blood medium as on the other media. Cultures from Case 3 liquefied both

Koch's and ox-blood media, those from Cases 1 and 2 did not; those from Cases 1 and 2 standing at cool temperature soon showed evidence of liquefaction.

*Dorsett's Egg Medium.*—Cultures from Case 1 grew better than those from Cases 2 and 3, but growths were neither rapid nor luxuriant as on other media.

*Milk.*—Growth showed as a bottom growth within 20 days, a falling down to the bottom of the tube of the solid constituents occurring, the supernatant fluid remaining as a clear upper stratum. No coagulation. In litmus milk the changes produced were the same as in ordinary milk; no change in color, no acid reaction after 20 days.

*Conradi-Drigalski Medium.*—No growth.

*Potato and Carrot.*—Growth occurred in from 3 to 4 days as dirty-white pinpoint moist colonies, doubling themselves in size in 3 days, while fine delicate mycelia were sent out over and into the media. Growth from the 7th day on was very rapid, and covered by a white fine downy mould-like formation; surface wrinkled quickly and was resistant to efforts at removal. On these media the growth strongly resembled that of an older growth in glycerin-agar tubes. Microscopic findings were the same as on other media.

*Fermentation of Saccharose, Maltose, Dextrose, Levulose, Lactose and Mannite.*—Cultures from Case 1 fermented all sugars with the production of CO<sub>2</sub> and alcohol for 2 generations. Inoculations into sugar media afterwards from agar growth after 5 months failed to ferment sugars; inoculations made 8 months afterwards from Koch's blood serum failed to ferment the media. Cultures from Cases 2 and 3 did not ferment any of the different sugar media tried.

*Gelatin.*—Growth occurred along the needle tract out and into the media as fine delicate rays. No liquefaction.

*Potassium Iodid 5% Solution in Broth and Glucose Broth.*—Growth was typical as on other liquid media, but the individual colonies were a trifle smaller.

#### ANIMAL EXPERIMENTS

Dogs 1 and 2, Cases 1, 2, and 3, were inoculated intraperitoneally with macerated agar growth, and pieces of growth on agar-agar were implanted subcutaneously. Reactive nodules appeared in from 4 to 6 days, finally enlarging to the size of a walnut; these nodules were tense and elastic, but did not fluctuate. The nodules diminished in size in from 2 to 4 weeks and then disappeared. Postmortem findings were negative.

Guinea-pigs 1, 2, and 3, Cases 1, 2, and 3, were inoculated with from 2 to 5 c.c. of bead-macerated broth cultures in physiologic salt solution subcutaneously and intraperitoneally. The animals also were exposed to a spray directed against the face.

Guinea-pig 1, Case 1, inoculated intraperitoneally, showed slight malaise in 5 hours and on the 2nd day a hot painful swelling occurred in the right testicle with great malaise. On the 11th day all evidences of scrotal trouble had disappeared. The guinea-pig resumed its normal habits and no other lesions were present. After 3 months the animal, on its back, showed a hard firm nodule, very itchy and situated just to the left of the median line. This nodule enlarged to the size of a hickory nut, becoming as it enlarged doughy and painful to the touch. The tumor-like mass covered the last dorsal and first 3 lumbar vertebrae. On the 5th day after appearing, the mass broke and discharged a glairy reddish-yellow pus, which upon examination in KOH solution showed round doubly contoured bodies. Inoculations were made into glucose-agar slants and the micro-organism obtained in pure culture. They grew by mycelial formation alone without any budding, and in 1 month had almost filled the tube. The

abscess healed completely and at no time during the second attack, the first being represented by the scrotal swelling, was the animal in poor spirits.

Guinea-pigs 2 and 3, Case 1, and Guinea-pigs 1, 2, and 3, Cases 2 and 3, showed slight reactive nodules in from 4 to 5 days, which soon disappeared. Findings after the animals were killed, were negative, including blood cultures.

Rabbits 1 and 2, Cases 1, 2, and 3, received subcutaneous and intraperitoneal inoculations of 2 and 5 c.c. of broth culture which had been shaken with beads, and Rabbit 2, Cases 1, 2, and 3, received 0.5 c.c. injections into the marginal ear vein. All rabbits except Rabbit 2, Case 1, showed negative findings. Rabbit 2, Case 1, showed with other rabbits a small tense nodule at the site of inoculation and slight malaise. In 4 months a hard painful itchy tumor-like mass was felt over the costal cartilages of the ribs. On the right side and located directly under the skin in the subcutaneous tissue, it seemed to be attached to the osseous structures underneath. This lesion was followed by a swelling of the left metatarsal region, which lasted only 10 days. A small hard palpable nodule was also found on the right ventral surface seemingly attached to the underlying muscles and fascia beneath. Appetite was still good; no nervousness or emaciation was present. At times the animal bit and scratched the areas described. The mass on the right side increased to the size of an apple. At the end of 5 months and 20 days Rabbit 2 was killed. The tumor-like mass had not ruptured and was still firm to the touch, with a fluctuating center. It was not attached to the underlying or overlying structures, but was situated in the subcutaneous areolar tissue and consisted macroscopically of a white fibrinous capsule, 1/8 inch in thickness, filled with a firm white cheesy mass, traversed by connective-tissue septa. The nodule on the abdominal fascia presented a similar picture. Spraying cultures into nostrils of all rabbits and guinea-pigs at intervals of from 2 to 5 days produced negative results.

*Microscopic Findings.*—The large tumor-like mass in the rabbit showed connective tissue septa enclosing a granular mass of large epithelioid cells and leukocytes with no micro-organisms present. Liver showed congestion with focal necrosis scattered throughout, but no micro-organisms. Kidneys were congested with beginning parenchymatous nephritis. Lungs showed small necrotic areas with the formation of giant cells; no micro-organisms present.

White Rats 1 and 2, Gray Rats 1 and 2, Cases 1, 2, and 3, each received intravenous and subcutaneous injections of 0.5 c.c.. Reactive nodules appeared at the sites of inoculation with slight malaise, recovery following in from 5 to 6 days. Upon 2nd inoculations the animals showed a dull listless attitude which lasted from 9 to 10 days. White mice 1, 2, and 3, Case 1, 2, and 3, received intravenous and subcutaneous injections with the same reactive results as in the case of the rats, only one mouse dying. Postmortem findings proved negative in all but Mouse 1, Case 1. In this mouse the lungs were studded with small gray nodules, which when fixed in formalin and embedded in paraffin, showed small foci of necrosis with invasion of leukocytes, epithelioid cells, and giant cells with partly disintegrated yeast-like bodies. Kidneys highly congested, showing slight parenchymatous nephritis. Liver highly congested. Spleen normal and other organs apparently normal. No cultures were obtained by inoculation from internal organs or blood. Specimens stained with eosin methylene blue, hematoxylin and eosin, and gram's stain.

#### SUMMARY

Summarizing the observations and experiments that have been given thus in detail, we find that the clinical history of the cases agrees with

that furnished by other investigators who have reported that living in basements or underground habitations predisposes to blastomycotic infection (Stober<sup>1</sup> and others). The primary lesions as found by Hektoen,<sup>2</sup> Montgomery,<sup>3</sup> LeCount and Bechtel,<sup>4</sup> and others, are identical with those described here and with those previously reported. Patients 1 and 2 complained of cough and severe pains in the chest, while Patient 3 gave the history of a pimple starting the cutaneous lesions. The only gross differences between the pure cultures were the spike-like prominences in those from Case 1 and their power to ferment sugar for 2 generations, while the organisms from Cases 2 and 3 possessed the power to liquefy blood serum. It is probable that the three organisms were identical, as most of the gross variations in the culture media observed in Case 1, appeared in the other two in time. This agrees also with the findings of Hamburger,<sup>5</sup> Montgomery and Ormsby,<sup>6</sup> and others. The aerial hyphae were more prominent in the spring and summer, while the rugose moist colonies were more common in the fall and winter. Growth by mycelial formation occurred most commonly, altho the incubator temperature favored a budding growth, but at no time in excess of mycelial formation. All growths were firmly incorporated into the solid media, so that it was necessary to break the culture media in order to remove the colonies.

Microscopic observations were made in the hanging drop in order to find out the method of reproduction, and it was in one instance possible to observe a growth with a projection of the protoplasm resembling a pseudopodium, which again in time formed a complete new mycelial segment. Granules and spherical ball-like particles as described by Ricketts<sup>7</sup> and others appeared in from 8 to 24 hours. After the formation of well-established lateral projections, nuclear-like bodies containing refractile and motile granules were observed, but no staining methods could demonstrate them later.

Beer-wort and nutrient-broth growths of all three strains were poured on gypsum blocks and kept at room temperature. The organisms were examined at the end of 12, 24, and 36 hours; after 24 hours the cultures from all cases showed round spherical endospores in the bud-like formation, numbering from 1 to 4 and 5 spore bodies. Tubes

<sup>1</sup> Arch. Int. Med., 1914, 13, p. 509.

<sup>2</sup> Jour. Am. Med. Assn., 1907, 99, p. 1071.

<sup>3</sup> Jour. Cutan. Dis., 1901, 19, p. 26.

<sup>4</sup> Arch. Int. Med., 1914, 13, p. 609.

<sup>5</sup> Jour. Infect. Dis., 1907, 4, p. 201.

<sup>6</sup> Arch. Int. Med., 1908-09, 2, p. 1.

<sup>7</sup> Jour. Med. Research, 1901, 6, p. 373.

of the organisms were sent to Mr. Richard Wahl<sup>8</sup> for examination as to spore-formation. The following report was received:

Cases 1, 2, and 3 were inoculated in hopped wort with a specific gravity of 1.0488, unhopped wort of the same specific gravity, and bouillon made the usual way and placed at 25 C. for 48 hours. At the end of this time, the hopped wort inoculated with Stems 1, 2, and 3, showed no signs of development. These samples were again examined after four days and showed no signs of development. The bouillon sample inoculated with Stems 1, 2, and 3 were examined after

TABLE 1  
DIFFERENTIAL FEATURES OF THE THREE STEMS OF ORGANISMS

Gross Cultural Appearances																																				
Source of Cultures																																				
																					Blood	Pus	Incubation period of cul- tures (Days)	Preferred Media	Solid Media								Liquid Media		Lit- mus Milk	Plates
																									Early	Later							Early	Later		
																										Elevated pinpoint	At first granular	Elevated	Wrinkled	Coherent and incorpo- rated with media	White	Dirty gray				
Herzog-Grant I	+	+	3-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+																
Grant-MacLane II	+	+	3-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+																
MacLane..... III	+	+	3-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+																

+ = affirmative.

- = negative.

± = inconstant or moderate.

? = not satisfactory.

48 hours and also after 4 days. They showed a development, but compared with the distillers' and brewers' yeast, the development was slow. The unhopped wort also developed slowly compared with brewers' and distillers' yeasts. This was the medium used for the endospore test. Three flasks of unhopped wort were inoculated with Stems 1, 2, and 3, and placed on gypsum blocks and placed at 25 C. An examination after 24 hours showed distinct endospores in all cases. Most of the cells contained one endospore, but some contained two and occasionally one contained three.

The behavior of the micro-organisms in saccharin and milk media would also indicate that they represent one species. Animal inoculations generally were not successful, tho others frequently have reported

<sup>8</sup> Biological Laboratories, Wahl Efficiency Institute for Baking Research, Chicago.

positive, fatal results. While inoculating rats intravenously, a point of a syringe came off, allowing a thick emulsion of micro-organisms to be precipitated into the eye of one of the assistants, and also a syringe point contaminated with blastomyces was introduced into the finger of an assistant accidentally, with no ill results in either case in one year from the time of the inoculations. A swelling of the testicle of a guinea-pig as reported by Hektoen also occurred in one of our animals,

TABLE 1—Continued  
DIFFERENTIAL FEATURES OF THE THREE STEMS OF ORGANISMS

Cultural Appearances		Morphology																								
Segmentation	Products	Spherical	Mycelial	Myceial diameter in microns	Spherical diameter in microns	Capsule	Protoplasm	Reproduction				Aerial	Thermal Limits													
								Tissue	Cultures, Surface and Submerged																	
									Budding	Hyphae with segmentation	Endospores															
Levulose	+++	+	+	4-6-8	10-30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannite	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CO <sub>2</sub>	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C <sub>2</sub> H <sub>5</sub> OH	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
In tissue	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
In culture	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
In tissue	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
In culture	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Finely granular	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coarsely granular	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vacuoles	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Small movable granules	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nucleus	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Endospores	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Budding	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hyphae with segmentation	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Endospores	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Budding	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hyphae with seg- mentation	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lateral or termin- al conidia	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lateral conidia	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ascospores	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Killed by freezing	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Killed by drying	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Death point	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pathogenicity to animals	+++	+	+	4-6-8	10-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

= to second generation.

= no information in our finding.

= forms large aerial Hyphae becoming smaller, disappears 4th generation.

but at no time was it as extensive as the reaction with *B. mallei*. A pure culture of blastomyces killed by heating at 65 C. for 1 hour, was rubbed into the scarified arm of a woman suffering from sporotrichosis, but no inflammatory or other changes occurred on the arm so treated. The agglutination test with this patient's blood serum also proved negative when tried with an emulsion of blastomyces.

The exact botanical classification of the organisms usually called blastomyces in medical literature is still doubtful. Whitman<sup>9</sup> in a contribution to the botany of the organism of blastomycosis, calls attention to this fact and asserts that he has been able to observe in a case of

<sup>9</sup> Jour. Infect. Dis., 1913, 13, p. 85.

fatal systemic blastomycosis, sporangium formation of the ascus, enabling him to properly classify the organism. The name blastomyces was first used by Gilchrist and Stokes, who were first to report an American case, and who had noticed the yeastlike character of the micro-organism and its tendency to multiply extensively by budding. Ricketts thought that the so called blastomyces properly belonged to the family Oidium, while Ophüls suggested the designation coccidioides or coccidioidal oidiumyces. Whitman in sections of the tissues from his case found groups of spherical bodies contained in a capsule consisting of a dense nonstaining membrane about 10 microns thick. The capsules were round or slightly oval and within them were found a large number (up to 50 or more) of round or slightly reniform bodies approximately 7 microns in diameter, lying closely packed together in the sac. He also claimed that places were seen where the ascus had ruptured and had discharged its spores into the neighboring tissues, where they had become enlarged with unequal rapidity, so that one could see spores of every size from the smallest to the largest. From these observations Whitman came to the conclusion that the organism of blastomycosis is an ascus-producing fungus, and he placed it according to the classification proposed by DeBeurman and Gougerot among the exoasaceae as a new genus, *Zymonema*, which are vegetable organisms of mixed characters, that is, yeast forms and mycelial threads. However, Whitman is compelled to confess that he has never been able to get any ascus-formation in his pure cultures, and he says:

Cultures from the case have been grown on various media and have been observed for about one year, without disclosing more than what other observers have recorded. One finds the mycelial threads septate and branching, at irregular intervals chlamydospores, conidiospores forming either at the end of the hyphae or in continuity, the conidiospores in chains and separated by septa, in which case they represent the oidium type of spore-formation. The conidiospores are at first elongated, later they become more and more spherical and are finally set free, to become apparently the yeast-cell bodies from which the organism earned its earliest name. It is worth noting that the conidia-bearing hyphae are larger and denser than the vegetative threads.

#### A CASE OF GENERAL BLASTOMYCOSIS IN A DOG

Dr. Maximilian Herzog furnishes the following statements concerning an incomplete observation on a case of undoubted general blastomycosis in a dog: A few years ago he received at the laboratory of pathology of the Chicago Veterinary College, a specimen of the lungs of a dog. It was said that this dog had been suffering from a wasting disease and had finally died from it and that the most marked changes

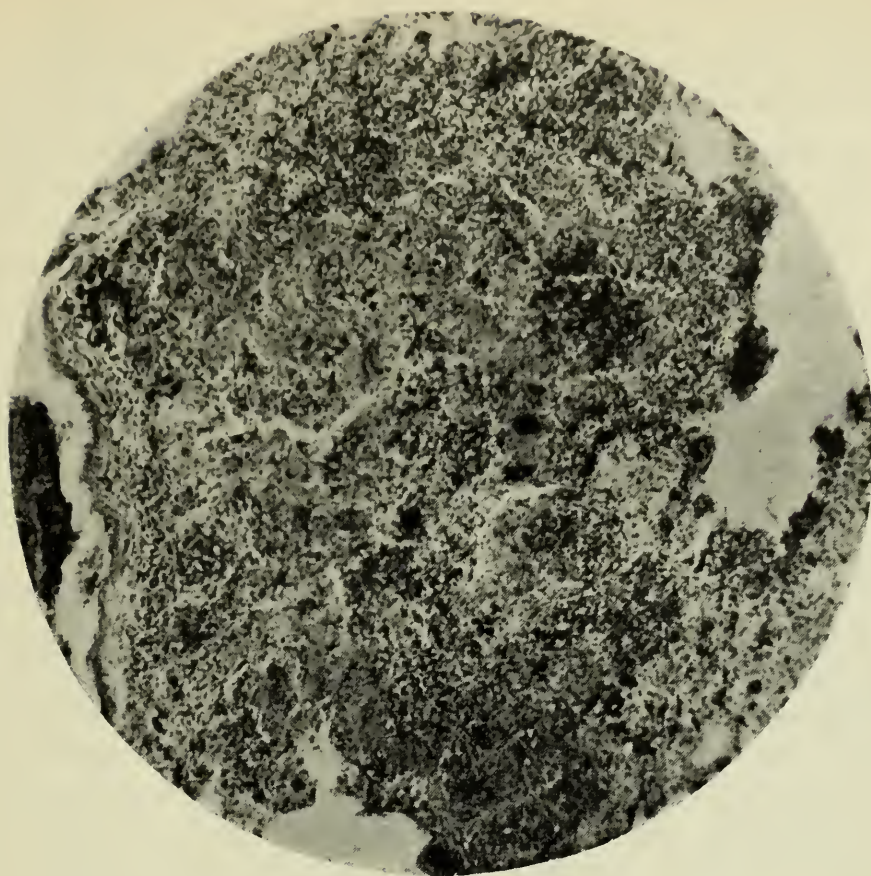


Fig. 8. Blastomycotic nodule in the lung of a dog, dead from generalized blastomycosis. Photomicrograph. Low magnification.

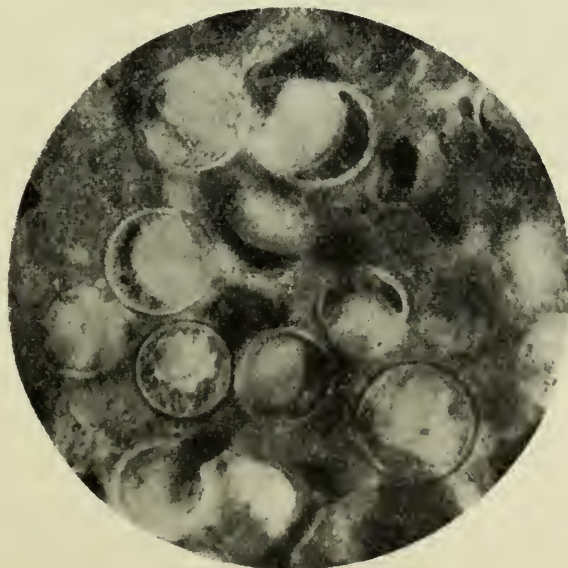


Fig. 9. Section from the nodule shown in Fig. 8. Photomicrograph, oil-immersion magnification, showing a number of double-contoured blastomyces.

upon postmortem examination had been found in the lungs. The latter when seen presented the picture of a miliary tuberculosis, the pulmonary parenchyma was riddled with grayish white opaque nodules from the size of a millet seed to a small pea. It was therefore believed that this was a case of canine miliary pulmonary tuberculosis. This condition, while not at all common, is however known to occur, and the specimen therefore was not immediately examined microscopically, but pieces of the pulmonary tissues were placed in alcohol or formalin for subsequent microscopic study. The latter was made only after the lapse of several months, when it was found that this was not a case of tuberculosis but one of blastomycosis. In the meantime the lungs from which the tissue had been taken had been lost and with them the name of the person who had sent in the lungs; hence nothing could be learned about the history of the case.

Microscopic examination of the sections showed the following: Under a low power the nodules noticed upon macroscopic inspection of the lungs appeared somewhat like tubercles, and the cells composing them seemed to be epithelioid and lymphoid cells. However, while the center of the nodules here and there was somewhat necrotic, there was nothing that looked like caseous material. There was one feature which distinguished the nodules, even under a low power, from tubercles; they were quite vascular. The nodules under low power also showed a considerable amount of coal dust.

Oil-immersion magnification showed in the nodules lymphoid and epithelioid cells, and also numerous polymorphonuclear leukocytes. The blood vessels were densely filled with red blood corpuscles, but the increase of leukocytes in the blood vessels of the nodules was relatively moderate. The most striking cellular element found in large numbers in the nodules was a spherical blastomyces, characterized by strongly marked double contours. The protoplasm of this micro-organism filled its capsule uniformly, and the crescentic appearance of the protoplasm as seen under the microscope and reproduced in the photomicrographs (Fig. 9) appeared to be largely due to an optical effect, and not to a real shrinkage of the protoplasm into the shape of a crescent.

# APPLICATION OF THE PURE-LINE CONCEPT TO BACTERIA \*

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In presenting this subject to geneticists we realize that much will be accepted by most of them, on the basis of their experience with higher forms of life, as more or less platitudinous. Nevertheless, since the facts with respect to bacteria may not be generally known to geneticists, and since the point of view which we shall present appears to be novel, or at least unappreciated, by most bacteriologists, we feel that a review and an interpretation of the facts in the light of present-day genetic advance will be of interest to both groups of investigators.

Variability in cultures was one of the most striking phenomena observed by the early investigators, and pleomorphism was commonly accepted by them. It was in fact the idea of early botanists, such as Nägeli and others, that "cocci could become bacilli, and bacilli, spirilla, as the chance of varying environment might dictate."<sup>1</sup> With the improvement of bacteriologic technic, however, the greater stability of bacterial forms became recognized; so that the definition of species and varieties, as well as of genera and higher groups, has been attempted after the manner employed in the classification of higher plants and animals. Nevertheless, the matter has always proved a vexatious one, owing to the great variability encountered, and as a result the classification of bacteria is still in a confused unsatisfactory state, and it is commonly very uncertain whether a particular form with which one may be working is the same or different from one which someone else has described. Much of the recent more careful work on variation has had as its object an improved classification, rather than the limits and causes of variation in themselves.

In their endeavor to delimit species bacteriologists have attempted to find the range in variation of their supposed species under a great

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<sup>1</sup> Winslow: The Systematic Relationships of the Coccaceae, with a Discussion of the Principles of Bacterial Classification, 1908.

variety of conditions, and because of the very small morphologic differences as compared with the physiologic plasticity, the latter has received the larger proportion of attention. In late years attempts have been made to apply the statistical method, particularly by Andrewes and Horder,<sup>2</sup> Bredemann,<sup>3</sup> Goodman,<sup>4</sup> Winslow,<sup>1</sup> Winslow and Walker,<sup>5</sup> Wolf,<sup>6</sup> and Buchanan and Truax.<sup>7</sup> Their studies have emphasized, what was already known from qualitative studies, that within recognized species there are distinct cultural races or varieties, each with its own characteristics and range of variability, and that these may exist side by side, independent of the environmental conditions. This belief is a distinct modification of the earlier view that one type could be transformed into the other directly by a change in the environment, or by selection.

As a result of his studies Winslow concludes that "bacterial types [species] should never be described on the strength of an examination of single individual strains; but only after a comparative study of the numerical frequency of each particular character in a considerable series of cultures." He lays down the following rules for classification: "First, each center of numerical frequency, marking a group of organisms varying about a distinct type in regard to a single definite property, may be recognized as a species. Second, those species which are bound together by the possession of a number of similar properties may be constituted as genera, and larger groups of genera, still characterized by some characters in common, may receive the rank of families or subfamilies." Winslow remarks that in the case of distinct bimodal curves "each mode may be considered a distinct type;" but he has apparently failed to consider the possibility that numerous distinct varying units, biotypes, may be masked in a unimodal curve.

#### TYPES AND CAUSES OF VARIATIONS

While, with respect to higher forms, there is coming to be a degree of unanimity as to the types of variation, even tho little may be known as to causes, the same can scarcely be said with respect to the bacteria. Altho a few recent investigators have attempted to bring order into this field, there has been a great deal of loose thinking and inconclusive

<sup>2</sup> *Lancet*, 1906, 2, pp. 708, 775, 852.

<sup>3</sup> *Centralbl. f. Bakteriöl.*, Abt. 2, 1908, 22, p. 44.

<sup>4</sup> *Jour. Infect. Dis.*, 1908, 5, p. 421.

<sup>5</sup> *Ibid.*, 1909, 6, p. 90.

<sup>6</sup> *Ztschr. indukt. Abstamm. u. Vererbungslehre*, 1909, 2, p. 90.

<sup>7</sup> *Jour. Infect. Dis.*, 1910, 7, p. 680.

reasoning on the subject, due, we feel sure, in most cases to a failure to appreciate the complexities and complications of the problem.

The most common fact in the experience of the bacteriologist is that variation occurs in cultures in response to changes in the numerous factors in the environment—temperature, light, composition and reaction of the medium, and the like. There is not absolute agreement as to the heritability of variations produced in this manner, but one may state that the majority of bacteriologists consider them at least partially heritable. In fact, it is among bacteriologists and paleontologists, of all biologists, that a belief in the inheritance of acquired characters still has its strongest hold. In the case of the bacteria, the belief is based on the not uncommon observation that when a particular condition produces a change in a culture of organisms, and cultivation is continued a sufficient time under the condition, the variation may become so thoroughly fixed that there is no return to normal, even tho cultivation under the original conditions is resumed. In other words, it is believed that the change has been impressed on the organism by the conditions of the environment, and such changes are called impressed variations.

It has been noticed from time to time, on the other hand, that distinctly different types may occasionally be plated out from a given culture, altho there has been no observable difference in the environment. Since the publication by de Vries of his observations on mutation in higher plants, increasing attention has been given to this subject with respect to bacteria, and many cases of "mutations" have been described.

The views as to bacterial variation most commonly held are presented by Winslow.<sup>1</sup> In discussing variation he says: "Among asexual forms there is no swamping effect of amphimixis to bring exceptional variations back to the specific mean. With unicellular organisms again there is no bar to the persistence of acquired characters. Bacteria respond in many ways to the direct influence of environmental conditions; and each strain transmits to a degree the impress of its recent history. Selection, too, has exceptional opportunities to modify these quickly reproducing forms. The immense number of generations which may succeed each other in a short space of time makes boundary lines as shifting as they would become among the higher plants if a dozen geologic epochs were considered at once."

A classification of variations\* is also presented, and later repeated in much the same form.<sup>5</sup> The following is compiled from both sources:

1. Observed differences in bacterial cultures, due to slight variations of the medium. These are considered as "not variations at all but temporary modifications of behavior due to the direct effect of existing conditions of cultivation;" this is not "real variation, altho in many cases it is mistaken for it."

2. There are 2 types of "true protoplasmic variations, which lead to characteristic differences in behavior under identical conditions." These are:

a. Variations "due to causes operating within the cells," arising "between the offspring of one original bacterium when growing under approximately the same conditions." In the absence of amphimixis this must be due to inequalities of nuclear division. Minute differences of this sort are "fluctuating variations," and others "well-marked sharply separated sports, or mutations." The former are common, the latter rare.

b. "Impressed variations" produced by the action of environmental conditions on a succession of bacterial generations. They are the impressions left upon an organism by its recent history; such as differences in virulence due to repeated animal inoculations, or the con-

\* There has been much confusion in the use of the term variation. Some bacteriologists have not considered ordinary fluctuations as variations at all, while others, as noted, speak of heritable differences only, as true variations, inferring that other differences are not. On the other hand, variations have been considered technically by some biologists as referring only to nonheritable modifications, as quoted from Darwin by the Century dictionary. This confusion has arisen in part from the fact that some have thought of variation as an act or process of modification, while more commonly we speak of any existing differences in the material considered, as variations. Confusion again arises from the fact that there may be variation of different orders, as for example, between individuals of the same clone, between different clones in the same culture, between different cultures, or in fact between any groups. We may also have variation of the same group at different times.

Biologists are coming very generally to consider any measurable differences as variations, but it is extremely important that the nature of the material being considered should be clearly defined. It is often difficult to do this. Thus intracolonial variation would probably ordinarily be understood to mean variation of the individuals composing a single clone; it might also be applied, however, to the differences between separate cultures derived from the same clone. Similarly intracolonial selection might be based on the selection of individual cells of a clone, or on the selection of separate colonies, or simply on the selection of ordinary transfers or mass cultures made from a given clone. There are other terms which may prove useful, but in each case it will be necessary to define clearly just what is meant. Thus interclonal variation would mean variation in different clones. These might, however, be mixed in the same culture, or be in separate pure cultures; and again interclonal selection would refer to the ordinary selection of clones from a mixed culture, or might mean selection from different cultures, each representing a pure clone. The terms intracultural and intercultural, referring to variation or selection, are descriptive of definite conditions or processes, but are less specific than the other terms suggested.

It should be noted that in the foregoing discussion of definitions, no mention has been made as to whether the variations concerned are heritable or nonheritable. This must be determined for each case. It is customary now to speak of nonheritable variations as fluctuations, fluctuating variations or modifications, and of heritable variations as such or as mutations. There is no fundamental biologic difference between what Jordan<sup>8</sup> terms environmental modifications and latent characteristics; both fall in the class that we have designated fluctuations or modifications.

<sup>5</sup> Proc. Nat. Acad. Sc., 1915, 1, p. 160.

verse adaptations of pathogenic forms to culture media. They may be due to direct action between the protoplasm and the environment (as in the example just given) or to selective force acting on fluctuating variations, presumably of the type mentioned under 2a.

Before discussing further the types of variation which probably occur, it will probably be best to outline briefly the pure-line concept and to discuss the results of certain investigators in its light.

#### THE PURE-LINE CONCEPT

The idea of biologic groups, such as species, or even smaller units, especially of nonconjugating (vegetatively propagating) or self-fertilizing forms, being in reality complex populations of a number of independent hereditary lines, is so familiar to biologists that it scarcely needs exposition.

A pure line is defined by Johanssen<sup>9</sup> as "the descendants from one single homozygotic organism, exclusively propagating by self-fertilization;" but for our purposes in the case of bacteria, in which reproduction is asexual, it may be taken as the descendants from any single cell. (The term clone is coming to be largely used by biologists to designate the aggregate line of descendants from a single individual when reproduction is vegetative or asexual. While the distinction is preferable on genetic grounds, it has seemed best not to employ the word in the present instance, since the general principle we are discussing is the pure-line concept whether applied to strictly pure lines, or to clones.) If a culture were started from 2 independent single organisms, successive transfers, unless 1 should be lost, would consist of 2 pure lines carried down side by side. While it cannot be accepted without proof, it seems fair to assume that if heredity is to be credited, the descendants of one of the cells should in the aggregate resemble more closely one another than they would the descendants of the other original cell, provided there was any hereditary difference in those two. There would thus be not only 2 pure lines of descendants, but 2 distinct genotypes, or hereditary complexes. If the hereditary composition of the two original cells were exactly the same, there would be but a single genotype, altho according to Johanssen's usage, there would still be represented 2 pure lines. In common usage, however, the terms pure line, genotype, and biotype have come to be used interchangeably to designate groups of organisms which are assumed to

<sup>9</sup> Am. Nat., 1911, 45, p. 129.

have exactly the same hereditary complexes, whether or not they are known to be descended from a single common ancestor.

The epoch-making researches of Johannsen<sup>9</sup> and of Jennings<sup>10</sup> have demonstrated the existence of distinct hereditary lines in self-fertilizing plants and in the protozoa, while subsequent work has shown that many of the phenomena of cereal-breeding may be referred to the same cause. An ordinary field of wheat, for example, tho it may all be considered a single variety, in reality consists of a mixture of hereditary types, or biotypes, each of which has its characteristic range of variation, and its own definite mode of response to environmental changes. In other words, the individuals of a given biotype, tho of the same hereditary nature, may exhibit a considerable range of variation in response to even slight differences in the environment. In this way, while the ranges of variation of two biotypes would not be entirely coincident, they might overlap to a considerable extent, and it would consequently be impossible to tell from the character of a single individual in this overlapping region whether it belonged to the one biotype or to the other. The only way to determine the matter would be to consider statistically a considerable number of descendants from the selected individual, for these should give the range of variation and mode characteristic of the type to which they belong.

In connection with the foregoing interpretation it is assumed that the environment does not ordinarily produce any change in the hereditary qualities of a biotype; so that variations produced by changed environmental conditions should have no permanent effect, the type returning to normal when normal conditions are restored. In recent years abundant evidence has been accumulated to prove the general truth of this assertion with respect to many animals and plants, and we shall see that it serves to explain also many of the phenomena observed in connection with bacteria. In practical cereal-breeding it has been found that often a single desirable individual may be selected which will breed true to its desirable qualities, so that all the desired results of selection are accomplished at a single stroke, and all that is necessary is to multiply the form by further propagation. This is the pure-line method of selection, in which the breeding value of an individual is judged on the basis of the character of its progeny.

We know, however, that mass selection is also productive of results, tho progress is much slower, and is often not permanent unless the selection is strictly continued. In mass selection, instead of separating

<sup>10</sup> Am. Nat., 1909, 43, p. 321; 1911, 45, p. 79.

out different biotypes at once, a number of lines are carried along at each selection, and as the more desirable ones predominate, and the less desirable drop out, progress is made. With this method it is very difficult to eliminate the undesirable biotypes entirely, and this explains why, when selection is relaxed, they may again rise to ascendancy. While, therefore, there has been an apparent change of the culture, or crop, as a whole, it has been apparent only, and not hereditary, except in so far as certain biotypes may have been dropped out. It must be borne in mind that changed environment or selection does not alter the hereditary qualities of the individual lines, tho it may eliminate or temporarily suppress some of them to the advantage of others.

Many bacteriologists have isolated and investigated strains of organisms which have remained true to characteristics different from those of the culture from which they were taken, yet few have grasped the full significance of the pure-line viewpoint as just outlined. Among those who have come most near to it may be mentioned Wolf<sup>6</sup> and Buchanan and Truax.<sup>7</sup> We shall now select a few cases to show how the pure-line concept furnishes an adequate explanation for facts not altogether harmonious on other views.\*

Dyar,<sup>11</sup> who made a careful study of *Bacillus lactis-erythrogenes*, poured plates of his organism and made cultures from isolated colonies, assuming that each consisted of the descendants from a single cell. Studies of the variability of the cultures obtained showed that most of them tended to return to the mean of the original culture. These results were taken to indicate the comparative permanency of species and races. It must be pointed out, however, that the presumption that single colonies are always, or perhaps even largely, the descendants of single cells, no matter how great the dilution, must be regarded with suspicion, and that the burden of proof lies on explanations based on results obtained by this method. Support of this contention is to be found in Dyar's own results later on, as he himself admits, and it is confirmed further by the recent investigations of Breed<sup>12</sup> on lactic-acid organisms (see also Drew<sup>13</sup>).

From certain of his cultures Dyar obtained a form which produced a crusty wrinkled growth in contrast to the normal smooth form. It seemed to appear suddenly, and he accordingly considered it a "sport or discontinuous variation." An agar-plate dilution was made from this crusted form and cultures taken from 125 isolated colonies; of these all but 3 came true to the new type, tho some cultures showed soft borders. The three were entirely of the normal soft form. The general result of succeeding tests was the same; that is, there always appeared to be a tendency toward a reversion to the normal type from the crusted form, but cultures from these reversions remained nearly true to

\*Since the writing of this paper there has come to our attention an extensive work by Toenniessen, "Ueber Vererbung und Variabilität bei Bakterien" (Biol. Centralbl., 1915, 35, p. 281), which cannot be reviewed at this time.

<sup>11</sup> Ann. New York Acad. Sc., 1895, 8, p. 322.

<sup>12</sup> Centralbl. f. Bakteriöl., Abt. 2, 1911, 30, p. 337.

<sup>13</sup> Bull. New York Agr. Exper. Sta., No. 373, 1914.

type, only 1 culture from 480 isolated colonies showing again the crusted form. The explanation of this apparent mutation from normal, reversion to normal, and mutation again to the crusted form, and also to a new intermediate type, is hinted at by Dyar himself. He says (p. 336): "It is conceivable that when the skinny growth of the wrinkly form is agitated in sterile water preparatory to making plates, that on account of its property of coherence, instead of becoming separated into single cells, certain of which are subsequently to develop colonies, the smallest portions really consist of masses of cells which might entangle a few cells of the smooth form, if this were originally present as a contamination. Thus the resulting colonies might be impure and the apparent phenomena of reversion be due to a separation of this mixture. Now the phenomena described above, of the soft borders to the wrinkly agar culture and colonies, are in favor of this view, and it is also observed that the growth when shaken up in water disintegrated with great difficulty." But as to the soft form, "a softer growth is scarcely to be imagined," and "in the hanging drop, the cells are seen singly, or, rarely, in pairs or short chains. It is, therefore, highly improbable that these should form masses and entangle a few of the wrinkly form."

Cultures of the same organism were exposed to high temperature (37.5 C.), and as is common in chromogenic bacteria, they tended to lose color and grow less vigorously. One culture became white around the edge; another grew paler and finally became spotted. Cultures from the white and yellow colonies showed distinct differences. Here again Dyar hit on an explanation which coincides with the pure-line concept. He concludes again that his cultures were mixtures, and that the white form was less injuriously affected by increased temperature; so that "the apparent effect of the temperature in producing a white form in the first instance may have really been due to a process of selection, the white [form] growing the faster and tending to supplant the other." "The variations toward a white form probably tend to become eliminated under natural conditions where the yellow form doubtless grows best. . . . It may be that this is, at least in great part, the true explanation of the production of the colorless races of chromogenic bacteria, which has been quoted as proof of the 'transmission of acquired characters.'"

It seems fair to conclude that Dyar was in reality working with a number of biotypes. The important question is, were these biotypes all represented in the original culture, or did they arise spontaneously or in response to environmental influences? Dyar's work does not permit of a conclusive decision on this point, tho he attempted to make his conditions as uniform as possible.

Goodman<sup>4</sup> developed 2 distinct forms of the diphtheria organism from 1 strain. One gave high acidity in dextrose broth and the other finally produced an alkaline reaction. The author concludes that there are no true distinguishing marks between the different forms, since the zymogenic power can be altered at will by artificial selection. He does not consider the possibility of his selections having separated 2 strains present in his original culture. This explanation must be considered, however, since we have seen from Dyar's results that the plating method is unreliable for separating single organisms.

Bredemann<sup>5</sup> studied statistically 27 cultures of *Bacillus asterosporus* isolated from soil samples from all parts of the world. Frequency curves showed dis-

inct modes which remained permanent for 3 years, yet there was no doubt that all were very closely related, and if they had all been put together the resulting mixed culture would no doubt have given the impression of considerable uniformity, unless means were taken to separate the different lines again.

Winslow and Walker<sup>5</sup> in a study of 2 strains of the paratyphoid bacillus, found that in spite of selection the frequency curves of the two remained distinct as to range and mode, altho the ranges overlapped. The difference between their results and Goodman's may be attributed to the fact that their two cultures, A and B, were true single biotypes to begin with, whereas his culture was mixed.

Buchanan and Truax<sup>7</sup> carried cultures of *Streptococcus lacticus* through 23 transfers, and attempted by selection to secure high- and low-acid-producing strains. Their efforts were unsuccessful. They decided that their failure was due to the fact that they were working with a pure line, within which selection was ineffective, and concluded that "the simplest method of securing high- and low-acid races of *S. lacticus* would be to select from a great number of sources in an effort to secure such races already established."

Rettger and Sherrick<sup>14</sup> were able by selection to secure divergence in color-production in certain chromogenic bacteria. They selected from portions of cultures which were the most or the least pigmented. As they themselves point out, their results may be due to a selection of already existing variations, rather than a production of variation by selection.

Hirschberg<sup>15</sup> reports negative results in an attempt to select for differences of shape in *Bacillus coli*.

Beijerinck<sup>16</sup> made extensive studies of variation and the effects of selection in bacteria, from which he drew far-reaching conclusions. In one case he reports profound physiologic modification of certain soil organisms which change nitrites to nitrates in the soil, and which thrive only in solutions of organic salts. By adding organic matter gradually in increasing amounts to the culture media, the organisms ultimately came to grow well in ordinary broth and lost entirely their power to grow in inorganic salt solution, or to oxidize nitrites to nitrates. From the phosphorescent organism *Photobacter splendidum*, isolated from the waters of the North Sea, cultures were obtained which lacked luminosity entirely, and others which were intermediate with respect to the light-producing function. The variations appeared in some cases to be reversible; luminous forms, for example, being obtained again from the dark mutant. These various changes Beijerinck classifies as mutations, submutations, modifications, and atavism, and discusses them in their relation to the "genenhypothese." One feels, however, that for genetic interpretation a much clearer analysis of the different forms and their origin is necessary.

The foregoing demonstrates that most bacterial cultures are mixed populations of a larger or smaller number of distinct hereditary forms or lines. Different cultural methods or other environmental changes act as a sieve through which the multiplex populations are sifted. Under a condition unfavorable to them certain biotypes become lost, or remain in a minority, while those forms flourish which are adapted

<sup>14</sup> Jour. Med. Research, 1911, 24, p. 265.

<sup>15</sup> Biometrika, 1913, 9, p. 331.

<sup>16</sup> Folia Microbiologica, 1914, 3, p. 91; 1915, 4, p. 15.

to that particular condition. Upon a return to normal the conditions are reversed, and the formerly suppressed types again assert themselves, provided the previous conditions have not been so severe as to kill them off entirely. It is just this chance which accounts for the inconstancy and lack of conformity of results of selection on mass cultures. Artificial selection under constant conditions would act in essentially the same way.

Many of the striking phenomena of bacteriology may be explained on this basis, particularly adaptations in bacterial cultures. As a special example may be mentioned the findings of Rosenow that streptococci isolated from a particular location or organ show a selective action for the same organ when introduced into a new host. Thus streptococcus cultures from stomach ulcers tend to produce stomach ulcers. Our explanation would be that the stomach environment, possibly the specific secretions, have tended to bring into predominance streptococcus biotypes especially adapted to that environment, and when they are inoculated into another animal, being the predominating forms they naturally grow most readily in the environment to which they are adapted, and in turn produce stomach ulcers in the new host.

Similarly, as Rettger and Sherrick have pointed out in speaking of the variations in virulence of pathogenic organisms, animal passage is "a mode of eliminating the avirulent individuals, and of encouraging the production of a pure pathogenic type."

#### THE EVIDENCE FOR MUTATIONS IN BACTERIA

We have seen that in the case of most of the results which have been put forward as examples of true hereditary mutations in bacteria the evidence of their really being such is inconclusive. They may as well be explained as the isolation of biotypes which were present, but not in evidence, in the original culture. The persistence of bacteria with little if any growth under unfavorable conditions is well known, and is illustrated by our own experiments on the persistence of lactic-acid organisms in highly diluted milk, in which the organisms remained in an apparently quiescent condition for long periods. The criterion of a mutation is that it must appear in a known pure line. It need not be of great magnitude or discontinuous in the sense that its variations may not overlap those of the parent form; but it must show a distinct racial modality of its own. Few experiments which meet these conditions have been performed, and they can be briefly reviewed.

Hansen<sup>17</sup> and Barber<sup>18</sup> each worked on cultures from isolated single cells. They used both yeast and bacteria. Hansen was able to develop physiologically typical cultures of bottom-fermentation yeast from top yeast, by cultivation of the progeny of single cells at a low temperature. He maintains that any variation which shows constancy must be taken as a mutation. His results with bacteria were somewhat similar. Barber obtained races of yeast showing morphologic differences by selection among the descendants from a single cell, but he estimated that the cells of the type which produced the new race occurred on an average of less than 1 in 5,000. From a culture of *Bacillus coli-communis* started from a single isolated cell he was able to produce a new race producing longer chains twice in 190 selections. The new race, while variable, showed constant difference from the parent form, and altho selection was practiced from each extreme of the new race, "there was neither accentuation of the peculiarity nor return to the original type." These would appear to have been true mutations and to have occurred spontaneously, as far as we can tell.

Wolf,<sup>9</sup> on the other hand, believes that he was able to induce mutations by change in environmental conditions, especially by the addition of certain poisons, such as corrosive sublimate, potassium bichromate, etc., to the culture media. Objection may be raised to Wolf's results on the ground that he depended upon the isolation of colonies from plate dilutions, yet it must be confessed he took extreme precautions. In the case of *Bacillus prodigiosus* he was apparently able to produce occasional mutations both of a lighter and of a darker shade of color than was to be observed in the original culture.

Jordan<sup>8</sup> recently reported what appears to be a perfectly clear case of mutation in *Bacillus coli*, which occurred under adequately controlled experimental conditions. A single cell was isolated by the Barber method from a freshly cultivated feces culture, and later from this 2 substrains were started from isolated single cells. These were carried through more than 500 transfers without showing any permanent change in their various characteristics, except that in one line a form appeared having the power of saccharose-fermentation. "This quality appeared in the seventh transfer on sodium-chlorid agar. It was not manifested by all the cells of the culture, but at the time of examination the saccharose-fermenting cells were greatly in the majority. On continuing the transfers they became the sole type found in the cultures, the nonsaccharose type disappearing altogether. . . . The newly acquired fermenting property has remained permanent throughout a series of over 500 test-tube generations (forty-eight-hour transfers), and is shown both by the cultures on sodium-chlorid media and by the strains transferred immediately on the aquisition of this property to ordinary nutrient agar and grown side by side with the two parent strains." In this case as in Barber's the mutation appears to have been spontaneous rather than induced.

#### PRACTICAL APPLICATIONS

If the interpretation here presented is established by future investigations, it will have a profound influence on practical bacteriologic methods, as the findings of Hansen, cited in the foregoing, have already had in the brewing industry. It is now customary to go beyond species

<sup>17</sup> Compt. rend des travaux du Lab. de Carlsberg, 1900, 5, p. 39. Abstracted in Centralbl. f. Bakteriöl., Abt. 2, 1901, 7, p. 439. Ibid., 1905, 12, p. 353.

<sup>18</sup> Sc. Bull. Kansas Univ., 1907, 4, p. 3.

lines, and to employ particular strains of bacteria for particular purposes, as, for example, in the manufacture of butter and cheese, while a particularly striking example is the use of a certain strain of the diphtheria organism, originally isolated by Park, for the manufacture of diphtheria antitoxin.

It has been very generally assumed that most of such strains have been produced by a modification of the typical organism, and that they are only relatively permanent, continual selection being necessary to keep them constant. Others, however, such as the diphtheria culture mentioned, have shown remarkable constancy under all conditions. In all probability the degree of constancy is directly correlated with the degree to which the strain approaches a single biotype or pure hereditary line, instead of being a mixture.

Should it be found that strains derived from single organisms show the high degree of constancy expected, the method of isolating lines in this way would be of great help in establishing cultures of any desired degree of virulence or other quality. Even then it would be necessary, of course, to guard against contamination, and also to be on the lookout for possible mutations which might occur within the culture. The procedure on the whole, however, would be more direct and more certain than the colony method of isolation.

#### CONCLUSIONS

There has been much confusion regarding variation in bacteria.

Most of the variation observed in ordinary cultures is due to the selection, more or less complete, by the investigator or by the environment, of particular pre-existent biotypes.

If the process of selection is complete, so that certain biotypes are completely lost, the culture will not return to normal type, even when placed again under normal conditions. Such a change is apt to be mistaken for a true hereditary mutation.

When the process of selection is incomplete certain biotypes are raised into predominance and others are suppressed, altho they may remain alive in the culture. In this case the return to normal conditions brings out the dormant types adapted to those conditions and the culture resumes its original characteristics. Such changes have commonly been referred to as modifications, tho they differ only in degree from those described in the foregoing.

The descendants of a single cell constitute a pure line, or, more strictly, clone, and on such material, altho there is variation, selection, is in no way effective unless actual hereditary mutations occur and are separated out.

Mutations apparently do occur in pure lines, both spontaneously and in response to certain environmental changes. Such mutations give rise to new races, or biotypes, which vary about modes of their own, and within which selection is ineffective.

Mutations may be of a magnitude to be readily appreciated, or they may be so small as to be determinable only by statistical methods.

The biotypes which make up the ordinary bacterial culture have undoubtedly arisen in times past as distinct mutations, and have lived on side by side, retaining their individuality because of the absence of amphimixis.

It is only by the most refined methods that the foregoing kinds of variations can be recognized and differentiated.

# DIFFERENT TYPES OF STREPTOCOCCI AND THEIR RELATION TO BOVINE MASTITIS \*

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Careful bacteriologic studies of many epidemics of acute tonsillitis have yielded convincing evidence that the infection may be milk-borne, and that the cause is a virulent hemolytic streptococcus. The source, however, of these organisms and the mechanism by which they gain entrance into the milk are still problems of vital importance in the understanding and control of the disease.

Many views have been advanced to explain the infection of milk with virulent streptococci, all of which are based on epidemiologic data. Some observers assert that streptococci of human origin are transmitted from man to man by means of milk contaminated in handling, while others consider mastitis or garget in cows (bovine streptococcal infections) to be the most important source of such epidemics. Altho much experimental work has been done on mastitis in cows, comparatively little attention has been paid to streptococcal udder infections and their relation to disease in man. Davis and Capps<sup>1</sup> have reported some experiments on bovine mastitis which have a direct bearing on the problem of the etiology of streptococco-tonsillitis. They were able to produce bovine mastitis by applying a culture of a hemolytic streptococcus to a fresh abrasion of the skin of the teat near the meatus. The tissue became infected locally and after several days signs of udder infection appeared. There was no caking or external sign of inflammation of the infected quarter, and the milk was not gargety, but it contained large numbers of leukocytes and streptococci. At the end of 4 weeks this condition had not changed. In another experiment a marked infection of the udder of several weeks' duration was produced by the injection into the milk ducts of a culture of a hemolytic streptococcus isolated from a case of tonsillitis. This mastitis was also evidenced by large numbers of leukocytes and bacteria in the milk. Capps and Davis concluded from these experiments that hemolytic streptococci of human origin may cause mastitis in cows, the organisms gaining entrance directly into the milk ducts or through infected abrasions of the teats. Furthermore, they demonstrated that mastitis may exist without the presence of any physical signs other than leukocytes and pathogenic streptococci in the milk.

In a recent review of the epidemiologic and bacteriologic data obtained from a study of several outbreaks of septic sore throat Smith and Brown<sup>2</sup> infer that the infection is of human origin and that streptococci from human sources are transmitted by means of milk from the infected udders of cows—udders in the milk ducts of which streptococci are growing and multiplying. Further they

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<sup>1</sup> Jour. Infect. Dis., 1914, 15, p. 135.

<sup>2</sup> Jour. Med. Research, 1915, 31, p. 455.

assume that the streptococci commonly associated with bovine mastitis are different from those of human tonsillitis and do not cause throat infections in man; and that the virulent hemolytic streptococci of human origin do not cause any appreciable inflammation of the cow's udder. On the other hand, they infer that streptococci of human origin may gain entrance into the udder and grow in the milk ducts, a condition which might explain epidemics of tonsillitis. Krumwiede and Valentine<sup>3</sup> also have recently advanced evidence in favor of such a view.

It is evident from a review of the literature that there is a question as to the susceptibility of the cow to infection with streptococci of human origin. Furthermore, little is known about the behavior of various strains of streptococci in the environment furnished by infected udders, particularly during long lapses of time. Do the differences between strains of streptococci of human origin and those of bovine origin represent changes due to environment? The following experiments have been designed primarily to throw light on these questions.

The technic used in these experiments was essentially the same as that used by Davis and Capps.<sup>1</sup> Human-blood-agar plates were used in the routine bacteriologic examination of the milk. The number of leukocytes was determined according to the Doane-Buckley method.

*Experiment 1.*—In this experiment a young healthy cow in a normal lactation period was used. The udder and teats were normal and well developed. The yield of milk per day was approximately 10 quarts. It contained from 20,000 to 40,000 leukocytes to the cubic centimeter, and the cultures were free from hemolytic streptococci. On April 29, 1915, 5 c.c. of a 36-hour litmus-milk culture of a hemolytic streptococcus were injected into the first quarter of the udder by means of a sterile glass syringe and rubber catheter. This strain of streptococcus had been obtained by Dr. D. J. Davis in 1912 from the udder of a cow suffering from mastitis. (Further details concerning this organism were published in the report of the Chicago milk epidemic by Capps and Miller,<sup>4</sup> and by Davis.<sup>5</sup>) After the quarter had been thoroughly cleansed and milked dry the catheter was introduced to a distance of 10 cm. from the meatus and the culture injected slowly. On the following day the udder was swollen, reddened, hot, hard, and tender. The little milk that could be expressed was curdy, stringy, and very yellow. The leukocytes numbered 1,900,000 to the cubic centimeter, and were principally of the polymorphonuclear type. Smears from the milk revealed numerous gram-positive encapsulated diplococci. The number of bacteria to the cubic centimeter was 27,000. On the second day the udder was markedly inflamed and caked, and the regional lymph glands were slightly enlarged and tender. The milk was gargety and teeming with hemolytic streptococci. This acute infection continued over a period of 10 days; then the leukocytes and bacteria began to decrease, and the physical signs of inflammation to subside. At the end of the first month examination of the milk revealed approximately 200,000 leukocytes and 3,000 streptococci to the cubic centimeter. The quarter very slowly decreased in size and the character of the milk did not change appre-

<sup>1</sup> Jour. Med. Research, 1915, 33, p. 231.

<sup>4</sup> Jour. Am. Med. Assn., 1912, 58, p. 1848.

<sup>5</sup> Ibid., p. 1852.

ciably until August 30, 1915, 124 days after infection. At this time there was an acute exacerbation of the infection lasting 3 weeks, after which a chronic course was again resumed, this condition continuing without noteworthy change up to the last observation (215 days after inoculation). The quarter decreased in size until at the end of the 215 days a very hard mass about the size of a hen's egg remained. From 1 to 5 c.c. only of a watery purulent material containing strings could be obtained on milking. The streptococci were still present in pure culture. It is noteworthy that the general condition of the cow did not change during the infection. Table 1 shows the numbers of leukocytes and of bacteria found in examinations of the milk at different times during the infection.

TABLE 1  
THE NUMBER OF LEUKOCYTES AND BACTERIA IN MILK FROM THE MASTITIS PRODUCED  
IN EXPERIMENT 1

Number of Days After Injection	Number of Streptococci per c.c.	Number of Leukocytes per c.c.	Number of Days After Injection	Number of Streptococci per c.c.	Number of Leukocytes per c.c.
0	0	38,000	50	470	179,000
1	27,000	1,900,000	60	445	187,000
2	40,000	6,400,000	70	830	161,000
3	47,000	12,800,000	80	580	142,000
4	88,000	18,500,000	90	780	122,000
5	50,000	19,400,000	106	1,220	202,000
6	96,000	12,000,000	124	4,200	2,600,000
7	45,000	10,300,000	138	11,000	9,800,000
8	51,000	9,100,000	156	9,200	2,800,000
10	30,000	7,340,000	169	1,100	310,000
15	38,000	4,700,000	177	7,300	180,000
20	47,000	1,240,000	188	7,800	410,000
25	22,000	560,000	195	9,600	220,000
30	1,320	193,000	205	7,100	400,000
40	240	167,000	215	3,100	270,000

*Experiment 2.*—A technic similar to that in the preceding experiment was used: 5 c.c. of a 36-hour litmus-milk culture of nonpathogenic nonhemolytic *Streptococcus lacticus* were injected into a normal quarter of the cow's udder on May 12, 1915. The following day this quarter was distended and tender. The milk was fluid but purulent in character. The leukocytes numbered 440,000 and the streptococci 600 to the cubic centimeter of milk. The streptococci were rather numerous in the stained smears. The next day the udder was markedly distended, hot and tender, and the milk contained a few stringy masses. On the 4th day the leukocytes numbered 4,600,000 and the streptococci 8,100 to the cubic centimeter; the milk was very yellow, purulent and stringy, and the regional lymph glands were enlarged and tender. After this the condition of the udder and the milk rapidly improved, until on the 21st day of the course there were no evidences of infection to be found on examination of the quarter or of the milk. The leukocyte count had returned to normal, and the streptococci had entirely disappeared from the milk. In this infection the course was acute and the recovery rapid. At no time was there any evidence of caking. After recovery the quarter functioned normally, the amount of milk per day being approximately the same as before the infection. There was no noticeable general reaction in the cow. The numbers of leukocytes and bacteria to the cubic centimeter of milk at different times during the infection are given in Table 2.

TABLE 2

THE NUMBERS OF STREPTOCOCCI AND LEUKOCYTES IN THE MILK AFTER THE INJECTION OF A CULTURE OF STREPTOCOCCUS LACTICUS INTO THE UDDER (EXPERIMENT 2)

Number of Days After Injection	Number of Streptococci per c.c.	Number of Leukocytes per c.c.	Number of Days After Injection	Number of Streptococci per c.c.	Number of Leukocytes per c.c.
0	0	48,000	10	750	96,000
1	600	440,000	12	620	72,000
2	8,500	528,000	14	320	61,000
3	7,300	4,200,000	16	210	75,000
4	8,100	4,600,000	18	92	49,000
5	3,200	1,700,000	19	7	53,000
6	2,460	940,000	21	0	46,500
7	1,800	720,000	24	0	42,000
8	1,150	400,000	30	0	51,000
9	1,600	240,000			

*Experiment 3.*—This experiment was undertaken as a control for the previous experiments. Three quarters of the udder of a normal healthy cow were carefully cleansed and milked. One quarter was then inoculated with 1 c.c. of a 36-hour litmus-milk culture of the strain of hemolytic streptococcus used in Experiment 1, another quarter with 1 c.c. of a 36-hour litmus-milk culture of *Streptococcus lacticus*, and a third quarter with 1 c.c. of sterile litmus milk. Twenty-four hours later the quarter injected with the hemolytic streptococcus was distended, reddened, and tender, the milk thick, purulent, and stringy, stained smears of which showed polymorphonuclear leukocytes and encapsulated diplococci. The leukocytes numbered 5,200,000 and the streptococci 3,400 to the cubic centimeter. The quarter inoculated with *Streptococcus lacticus* was somewhat distended and tender, and the milk yellow, syrupy, and acid. There were no strings but after the milk had stood awhile, a heavy sediment settled to the bottom of the tubes. The leukocytes numbered 320,000 and the bacteria 16,000 to the cubic centimeter. The other injected quarter was apparently normal in every respect after 24 hours. The infections with the hemolytic streptococcus rapidly became severe; on the 4th day the udder was hot, tender, and caked, the regional lymph glands enlarged and tender, and the milk gargety. The leukocytes numbered 6,800,000 and the bacteria 68,000 to the cubic centimeter. After this the inflammatory reaction gradually subsided and the infection assumed a chronic course similar to that of the infection in Experiment 1; 169 days after inoculation, the udder was small and very hard, the milk stringy and somewhat watery. The leukocytes numbered 132,000 and the bacteria 290 to the cubic centimeter. These streptococci were found in the milk in pure culture.

The quarter injected with the cultures of *Streptococcus lacticus*, on the 2nd day was distended and hot, but showed no evidence of caking. The milk was purulent and lumpy. The leukocytes numbered 560,000 and the bacteria 22,000 to the cubic centimeter. All signs of inflammation then rapidly disappeared so that on the 12th day the quarter was again normal; the milk was free from streptococci and the leukocytes numbered 47,000 to the cubic centimeter. At no time during this infection were there any evidences of caking, and recovery was rapid and complete. The quarter injected with litmus milk remained unchanged. The differences in the result of the 3 inoculations are well shown in Table 3.

TABLE 3  
THE NUMBER OF LEUKOCYTES AND OF BACTERIA IN THE MILK AT DIFFERENT INTERVALS  
FOLLOWING THE INOCULATIONS IN EXPERIMENT 3

Days After Inocu- lation	Hemolytic Streptococcus		Streptococcus Lacticus		Sterile Litmus Milk	
	Number of Bacteria per c.c.	Number of Leukocytes per c.c.	Number of Bacteria per c.c.	Number of Leukocytes per c.c.	Number of Leukocytes per c.c.	Number of Bacteria per c.c.
0	0	32,000	0	42,000	45,000	0
1	3,400	5,200,000	16,000	320,000	41,000	0
2	51,000	6,800,000	22,000	560,000	39,000	0
3	64,000	7,200,000	19,200	450,000	42,000	0
4	68,000	6,800,000	9,100	260,000	44,500	0
5	19,400	5,600,000	3,200	180,000	37,500	0
6	14,200	4,500,000	1,400	124,000	41,000	0
7	8,200	4,100,000	640	98,000	39,000	0
8	4,750	2,900,000	220	63,000	40,200	0
9	2,260	1,800,000	112	48,000	41,000	0
10	1,520	2,300,000	62	45,000	.....	0
12	1,140	1,340,000	10	47,500	47,500	0
12	1,250	1,420,000	0	47,000	41,000	0
13	1,060	1,500,000	0	36,000	37,000	0
14	1,110	1,380,000	0	43,500	41,000	0
15	1,260	1,220,000	0	25,600	40,000	0
16	2,100	970,000	0	44,200	42,000	0

*Experiment 4.*—Two cubic centimeters of a 36-hour litmus-milk culture of a strain of streptococcus isolated from man were injected into a normal quarter of the udder of a healthy cow after it had been milked dry. (This organism had been isolated by Dr. D. J. Davis from the peritoneal exudate of a fatal case of acute streptococcal peritonitis following an attack of septic sore throat during the Chicago milk epidemic in 1912.) On the following day the udder was tender and hot but there was no evidence of caking. The milk was thicker than normal; the leukocytes numbered 872,000, and the streptococci 330 to the cubic centimeter. On the 2nd day this quarter of the udder was hot, somewhat reddened, tender, and caked in the lower portion. Three days after inoculation, the udder was caked and the milk gargety. The leukocytes numbered 6,430,000, and the bacteria 1,210 to the cubic centimeter. The acute inflammation persisted for about 30 days, after which the quarter began to decrease slowly in size. At the last observation, however, 146 days after inoculation, the milk was still gargety and contained large numbers of hemolytic streptococci. The character of the milk and the number of leukocytes and of bacteria remained practically unchanged during the sub-acute stage of the infection. The general condition of the cow was at no time affected. Table 4 shows the observations made on the milk at different times during the infection.

TABLE 4  
THE NUMBER OF LEUKOCYTES AND OF STREPTOCOCCI IN THE MILK EXAMINED IN EXPERIMENT 4

Number of Days After Injection	Number of Streptococci per c.c.	Number of Leukocytes per c.c.	Number of Days After Injection	Number of Streptococci per c.c.	Number of Leukocytes per c.c.
0	0	41,000	61	10,000	6,100,000
1	330	872,000	73	12,200	8,800,000
2	860	3,640,000	88	10,600	2,300,000
3	1,210	6,430,000	100	6,000	7,200,000
6	3,800	16,300,000	108	7,800	5,700,000
10	10,300	18,200,000	119	13,000	8,200,000
16	16,100	14,210,000	126	6,800	8,700,000
26	11,100	9,500,000	136	4,700	3,600,000
47	7,300	6,800,000	146	9,700	2,800,000

*Experiment 5.*—In this experiment a nonpathogenic hemolytic streptococcus from milk was used. Two cubic centimeters of a 36-hour litmus-milk culture were injected into a normal quarter of a cow's udder after it had been milked dry. Twenty-four hours later this quarter was distended and tender. The milk was purulent, the leukocytes on the second day numbering 6,200,000 and the streptococci 37,000 to the cubic centimeter of milk. After this the condition of the milk rapidly improved, the number of leukocytes reaching normal on the 10th day. On the 16th day the streptococci had entirely disappeared. At no time during the experiment were there any signs of caking or lymphadenopathy, and the milk was gargety only for a period of 4 days. The numbers of leukocytes and of bacteria to the cubic centimeter of milk at different times during the infection are given in Table 5.

TABLE 5

THE NUMBER OF LEUKOCYTES AND OF STREPTOCOCCI IN THE MILK FROM THE MASTITIS PRODUCED IN EXPERIMENT 5

Day of Injection	Number of Streptococci per c.c.	Number of Leukocytes per c.c.	Day of Injection	Number of Streptococci per c.c.	Number of Leukocytes per c.c.
0	0	32,000	8	3,100	93,000
0	0	32,000	9	2,600	69,000
1	8,100	4,800,000	10	2,920	43,000
2	37,000	6,200,000	11	310	37,000
3	17,000	4,200,000	12	480	39,100
4	24,000	2,900,000	13	170	34,000
5	13,200	870,000	14	62	36,500
6	9,700	122,000	15	0	42,000
7	4,600	89,000	16	0	37,500

In these experiments a definite inflammatory reaction was produced in the udders of the cows by the injection of cultures of 4 different types of streptococci. In Experiment 1 the organism used was isolated from bovine mastitis and conformed generally in cultural and morphologic characteristics with what is described by Davis,<sup>5</sup> Smith,<sup>2</sup> and others, as the human type of the hemolytic streptococcus. When a culture of this organism was injected into the milk ducts of a cow a very severe garget resulted, which persisted in a chronic atrophic form up to the last observation, 215 days after inoculation. In two instances (Experiments 2 and 3) cultures of *Streptococcus lacticus* were introduced into the udder and an acute inflammatory condition of the milk ducts followed. This inflammatory reaction rapidly subsided, however, leaving the udder unchanged. The output of milk before and after the infection was approximately the same. As a control for these experiments sterile litmus milk was injected into one quarter of the udder with no evidences of any consequent inflammatory change. In Experiment 4 a strain of a hemolytic streptococcus isolated from the peritoneal cavity in a fatal case of acute tonsillitis was used. The

injection of 2 c.c. of a 24-hour litmus-milk culture of this organism produced a severe udder infection similar to that in Experiment 1. The quarter was hard and caked and the milk gargety. The amount of milk obtainable from the infected quarter was greater than in Experiment 1. In other words, the function of the gland was not so markedly altered. Following the acute stage of the infection the gland began to decrease in size until the last observation, 146 days after injection, when only from 30 to 50 c.c. of purulent milk could be obtained from this quarter daily. In another instance an acute inflammation of the milk ducts was produced by the injection of a litmus-milk culture of a nonpathogenic hemolytic streptococcus of the milk type (Experiment 5). This organism, which had been isolated from normal milk by Dr. Davis, corresponded in every detail with what he describes as the milk type of hemolytic streptococcus.<sup>6</sup> The infection, which was of short duration, was evidenced by an increase in the number of leukocytes and of streptococci in the milk, without signs of caking. The udder was normal after the infection had subsided. The clinical picture of this mastitis was similar in every detail to that observed when nonhemolytic *Streptococcus lacticus* was used. On the basis of the observations of Davis,<sup>5</sup> Smith,<sup>2</sup> and others, it seems reasonable to assume that this nonpathogenic hemolytic streptococcus was of bovine origin. So mastitis has been produced in 6 instances by the use of streptococci both of human and of bovine origin, and the human types of streptococci found uniformly more virulent for the cow than the streptococci derived from bovine sources. It is interesting to note in connection with these observations that the quarters of the udder are entirely separate; one or more quarters may be infected while the others remain normal. Again, in all the infections bacteriologic examination of the milk usually yielded pure cultures of the invading organism.

Another important factor in streptococcal infections of the udder emphasized by these experiments is the length of time that the organisms may be found in the milk. Zschokke<sup>7</sup> found that bovine streptococci could be isolated from the milk of a cow 6 months after infection. Savage<sup>8</sup> found human streptococci in the milk of goats 219 days after their injection into the udder.

Similar results were obtained in this work when streptococci with characteristics of the human types were used and at the last observation there were no evidences that any of these experimental

<sup>6</sup> Jour. Infect. Dis., 1916, 19, p. 236.

<sup>7</sup> Schweiz. Arch. f. Tierheilk., 1897.

<sup>8</sup> Milk and Public Health, 1912.

infections were subsiding. The three infections were, at the writing of this report, respectively 215, 186, and 146 days old.

Different types of streptococci of human origin may cause mastitis if they gain entrance into the milk ducts. Furthermore, this mastitis may be so severe that the clinical picture simulates that of garget, and it may continue over so long a time that an atrophy of the mammary gland will result. The strain of *Streptococcus lacticus* and the hemolytic milk streptococcus used in these experiments may give rise to a definite acute inflammatory change in the milk ducts of a very transitory nature. The conclusion of Davis,<sup>5</sup> Smith,<sup>2</sup> and others, that virulent streptococci may be found in the milk ducts without any appreciable signs of inflammation externally, also seems to be firmly established by the following observations. One of the cows obtained for this experimental work was pronounced in perfect health by a competent veterinarian. Examination of the udder revealed no signs of inflammation or abrasions of the teats. The milk was normal upon inspection but in one quarter of the udder, examination for leukocytes and bacteria revealed signs of infection. There was a leukocytosis (from 150,000 to 200,000 to the cubic centimeter), and blood-agar-plate cultures made from the milk contained numerous hemolytic streptococci. The udder, the milk, and the general condition of the cow were apparently normal and these conditions did not change throughout the period of observation, lasting 2 months. The streptococci were arranged in pairs and short chains, nonencapsulated and gram-positive. They grew as small, translucent, slightly moist, grayish-white colonies on blood-agar plates with a clear transparent hemolytic zone from 2 to 4 mm. in diameter; produced a slight precipitate in ascites-dextrose broth, and fermented dextrose, lactose, saccharose, maltose, and salicin. They produced arthritis in rabbits in doses of one blood-agar-slant culture. The other quarters of the udder were normal in every respect—the milk free from hemolytic streptococci and the leukocytes varying between 20,000 and 40,000 to the cubic centimeter. From the description of this organism it would be considered as belonging to the human streptococcal group.

In these experimental infections the milk was markedly purulent and stained smears showed that phagocytosis of the invading organisms was much the more marked in the infections caused by nonpathogenic milk types of streptococci. In those caused by the hemolytic streptococci derived from human sources there was no appreciable phagocytosis, altho the streptococci were numerous in the stained preparations.

OBSERVATIONS ON THE MORPHOLOGY AND CULTURAL CHARACTERISTICS  
OF DIFFERENT TYPES OF STREPTOCOCCI DURING THE COURSE  
OF MASTITIS IN COWS

Streptococci derived from human sources during epidemics of septic sore throat are somewhat variable in their morphology and cultural characteristics. Such organisms are also in most instances highly virulent for rabbits, a characteristic which distinguishes them from strains of hemolytic streptococci associated with bovine mastitis. There is no convincing evidence that streptococci from bovine sources are the causative factors in epidemic tonsillitis, but the possibility that these organisms may acquire the morphologic, cultural, and pathogenic characteristics of the human streptococcal types cannot be denied.

TABLE 6

OBSERVATIONS ON THE MORPHOLOGY AND CULTURAL CHARACTERISTICS OF THE BOVINE STREPTOCOCCI ISOLATED FROM THE MASTITIS PRODUCED APRIL 29, 1915 \*

Day of Mastitis	Colonies on Blood Agar	Arrangement and Shape	Cap- sule	Hemolysis (Diameter of Zone, mm.)
0	Small, gray, dry.....	Pairs and short chains slightly oblong	+	4-6
27		Pairs slightly oblong.....		4-6
36				4
47	Small, round flat, gray, dry.	Pairs.....	+	4-6
69				4
81				4-6
92				6-8
106	Small, dry, slightly elevated.		Frag- ment	8-10?
124				6-8
127				8
136				6-8
148	Small, dry, and gray.....	Pairs and short chains.....	0	4-6
157				3-5
169				3-6
177				3-4
188			+	3-4
195				3-4
205				3-4
215				3-4

\* The table of observations for the same organism from the mastitis produced May 26,

Rosenow<sup>9</sup> has been able to modify the common *Streptococcus pyogenes* by growing it in unheated cow's milk. According to his results, in this medium these streptococci acquired all the cultural and morphologic characteristics of the encapsulated organisms commonly associated with septic sore throat. These modifications were accentuated by animal passage, but after prolonged cultivation on common artificial media they were lost.

In view of these observations the relation of different types of streptococci to the udder as regards virulence, growth, and ability to become modified therein, becomes a very important problem in the

<sup>9</sup> Jour. Infect. Dis., 1912, 11, p. 338.

study of the etiology of epidemic tonsillitis. For the study of this phase of the problem the hemolytic streptococci isolated at frequent intervals from the different udder infections here described were carefully studied morphologically and culturally, with results as recorded in Tables 6 and 7.

The streptococcus used in Experiment 1 grew on human-blood-agar plates in small dry gray colonies with a sharply defined zone of hemolysis from 4 to 6 mm. in diameter. The organisms were small, gram-positive, slightly oblong, and they occurred in pairs and short chains. There was usually a suggestion of a small narrow capsule around each of them. They acidified and coagulated litmus milk and fermented dextrose, saccharose, lactose, maltose, and salicin. They were killed by an exposure to 130 F. for 30 minutes. Doses of one common blood-agar-slant culture produced multiple arthritis and death in rabbits in from 3 to 5 days.

TABLE 6—*Continued*

OBSERVATIONS ON THE MORPHOLOGY AND CULTURAL CHARACTERISTICS OF THE BOVINE STREPTOCOCCI ISOLATED FROM THE MASTITIS PRODUCED APRIL 29, 1915 \*

Gram Stain	Growth in Litmus Milk	Growth in Plain Broth	Thermal Death Point	Sugars							
				Dextrose	Saccharose	Lactose	Maltose	Inulin	Salicin	Raffinose	Mannite
+	Acid and Coag.   Acid	+	130 F.	+	+	+	+	—	+	—	—
										+	
										±	
										—	
										+	
										—	
			130 F.								

1915, is not included in this paper as the findings were identical with those shown in Table 5.

At the last observation this streptococcus was still growing in the infected udder after 215 days and no appreciable change in any of its characteristics had been noted, save a slight increase in capsular substance and a slight variation in the hemolytic power. After the onset of the mastitis the organisms in the infected milk were surrounded by well-defined capsules. The capsules were further developed by passing the organisms through rabbits, but when the organisms were transferred to artificial media the capsules disappeared. The cultural characteristics, save some variation in the hemolytic power, did not change.

During the course of this infection a second organism appeared suddenly. On the 136th day of the infection the blood-agar-plate cultures revealed the presence of a green-producing nonhemolytic streptococcus. The colonies were

very small, white, somewhat moist, and surrounded by a marked green halo. The organisms were medium-sized round and oval micrococci arranged in pairs and chains. They showed no capsules. They grew very slowly on blood agar, produced a homogeneous clouding in ascites-dextrose broth, and seemed to grow more luxuriantly under partial anaerobic conditions. They showed no growth in plain broth, acidified but did not coagulate litmus milk, and fermented dextrose, saccharose, lactose, maltose, salicin, and inulin. The organism was fatal for rabbits only in doses as large as the amount of sediment from 15 c.c. of an ascite-dextrose-broth culture. These streptococci gradually decreased in number until they disappeared from the milk on the 157th day of the infection. In a normal quarter of the udder used as a control for these studies a similar occurrence was twice noted. During the routine examination of the milk an avirulent green-producing streptococcus would appear in the cultures, to disappear a few days later. From this observation on normal milk, as well as from the cultural characteristics—low virulence and rapid disappearance—it seems probable that this avirulent streptococcus was a contamination that had gained entrance to the milk ducts through the teat.

The human type of streptococcus (Table 7) used in Experiment 4 differed somewhat from the organism used in Experiment 1. The colonies on blood-agar plates were large, moist, grayish-white, and spreading, often on the surface almost covering the clear hemolytic areas around them. The organisms were gram-positive, arranged in pairs and short chains, small, and each surrounded by a well-defined capsule. They acidified and coagulated litmus milk and fermented dextrose, saccharose, maltose, lactose, and salicin. They were killed by 30 minutes' exposure to 125 F. In doses of 1 blood-agar-slant culture they were fatal for rabbits, producing suppurative arthritis. During a period of 146 days in the infected udder there was no noteworthy change in the morphology or cultural characteristics of this organism. In the two infections the virulence of the organisms was increased slightly by growth in the infected udders. Small amounts of the cultures, injected into rabbits, produced arthritis constantly, and occasionally changes in the kidneys and myocardium.

Bacteria growing in the infected udder of a cow are subjected to life processes which tend to destroy them, and under such conditions variations in minor cultural or morphologic characters might be expected. Changed reaction of the media, the presence of numerous polymorphonuclear leukocytes, the immediate products of their disintegration, and other influences represented by inflammatory processes, are all factors which might affect the growth and morphology of bacteria. But in these observations no definite changes were noted in the growth or morphology of the different types of streptococci causing the udder infections. Organisms virulent for man seem to possess many morphologic and cultural characteristics which are recognized only when the bacteria are cultivated under certain environmental conditions. Capsule-formation and hemolysis are characters which almost

all types of streptococci seem to possess, but only under certain environmental influences can they be clearly demonstrated. Thus, as Rosenow and many others have observed, the ordinary *Streptococcus pyogenes* may develop a capsule on passage through animals, but on continued growth on artificial media this capsule disappears. In other words capsule-formation in the *Streptococcus-pyogenes* type might be considered a true characteristic but a variable one. Likewise, virulent green-producing streptococci seem to possess hemolytic power to a limited degree, which can be developed by prolonged cultivation on artificial media. Neither of these characteristics, however, in some types of streptococci can be made to disappear by varying of environmental conditions. Such organisms may be looked upon as definite types in respect to these persistent characters. The streptococcus used in Experiment 4 has been grown on artificial media since 1912, and has retained its capsule and mucoid growth, two characteristics which may be considered as fixed to a certain degree. The streptococcus used in Experiment 1, on the other hand, develops a capsule only under certain conditions, and it seems logical to consider this modification as a variation rather than a transformation into the type of streptococcus used in Experiment 4. Whether or not such variable characters in virulent streptococci can be made permanent remains to be demonstrated. It is most important in any study of bacteria isolated from infections in man to appreciate the wide variability which may be found in the morphology and cultural characteristics of these virulent organisms.

During the course of this work two udder infections produced by the injection of cultures of nonpathogenic *Streptococcus lacticus* were studied. This organism showed no changes in morphology or cultural characteristics throughout the course of these infections. The colonies on blood-agar plates were small, round, grayish-white, and surrounded by a green halo. Microscopic examination of the blood agar beneath and immediately around the colonies revealed disintegration of the erythrocytes. Where the blood agar was very thin there was some hemolysis, characterized by a narrow hazy zone around the colonies, such as is commonly observed in pneumococcus cultures. The green-production on blood agar is probably associated with a minute degree of hemolysis. This point has been emphasized because it might explain the observation made by Heinemann that green-producing streptococci of the lacticus type may become slightly hemolytic under certain environmental influences.

## SUMMARY

Hemolytic streptococci of human origin produce mastitis in cows, as Davis and Capps<sup>1</sup> have shown, when injected directly into the milk ducts. This mastitis may be severe, leading to a caked bag and later to a chronic inflammatory condition which results in an atrophy of the mammary gland. On the other hand, virulent hemolytic streptococci may grow and multiply in the milk ducts of a cow without causing any visible changes in the udder. The milk, however, as these observations show, contains hemolytic streptococci and an increased number of leu-

TABLE 7

OBSERVATIONS ON THE MORPHOLOGY AND CULTURAL CHARACTERISTICS OF THE HUMAN TYPE OF HEMOLYTIC STREPTOCOCCUS FROM THE MASTITIS PRODUCED JULY 11, 1915

Day of Mastitis	Colonies on Blood Agar Plates	Arrangement and Shape	Cap-sule	Hemolysis (Diameter of Zone, mm.)
0	Small, gray, moist, and spreading...	Diplococci and short chains...	+	2-4
6	Small, gray, very moist.....	Diplococci mainly.....		3-6
26	Spreading and moist.....			1-3
47	Spreading and moist.....			2-4
61	Irregular edges, gray, moist.....			1-3
73	Large spreading moist colonies.....			1-3
88	Spreading moist colonies.....	Diplococci and short chains...		1-3
100	Large, gray, very moist.....	Chains 4-10 organisms.....		1-3
108	Tend to spread, very moist.....			2-5
119	Large, spreading, moist.....			2-5
126	Dense center, very moist.....	Diplococci and long chains....		2-5
136	Large, gray, spreading, moist.....			2-4
146	Medium-sized, gray, moist.....			2-4

kocytes. These infections may persist over long periods of time in the form of a chronic mastitis.

*Streptococcus lacticus* of the type used in these experiments produces a very acute inflammation of the udder when cultures are injected directly into the milk ducts. This infection in my experiments was of short duration and left the gland functionally unchanged.

A nonpathogenic hemolytic streptococcus of the type commonly found in normal milk may give rise to a transitory inflammation of the udder when injected directly into the milk ducts, producing a mastitis similar in every detail to that produced by nonhemolytic *Streptococcus lacticus*.

The presence of pathogenic streptococci and an increased number of leukocytes in milk is indicative of a mastitis, and may be the sole indication of mastitis.

The quarters of a cow's udder under experimental conditions are apparently separate as regards infection. One quarter may be infected, while the others remain normal. Examination of the milk from each quarter of the udder is necessary before mastitis can be excluded in a suspected cow.

In 3 instances of bovine mastitis, all of which were due to hemolytic streptococci with all the characteristics of the human types, no noteworthy changes in the morphology or cultural characteristics of the invading organisms were observed in frequent examinations of the milk throughout the course of the infections. The distinguishing char-

TABLE 7—*Continued*

OBSERVATIONS ON THE MORPHOLOGY AND CULTURAL CHARACTERISTICS OF THE HUMAN TYPE OF HEMOLYTIC STREPTOCOCCUS FROM THE MASTITIS PRODUCED JULY 11, 1915

Gram Stain	Growth in Litmus Milk	Growth in Plain Broth	Thermal Death Point	Carbohydrates							
				Dex-trose	Saccha-rose	Lac-tose	Mal-tose	Inu-lin	Sali-cin	Raffi-nose	Man-nite
+	Acid and Coag-ulated	+	125 F.	+	+	+	+	—	+	—	—
			120 F.								

acteristics primarily noted for each organism were still present at the last observation, and there were no modifications which might be considered as indicating a change from one type to the other.

The cultural and morphologic characters of *Streptococcus lacticus* and of the hemolytic streptococcus derived from normal milk did not change during the course of the udder infections which they induced.

# HEMOLYTIC STREPTOCOCCI FOUND IN MILK

## THEIR SIGNIFICANCE AND THEIR RELATION TO VIRULENT STREPTOCOCCI OF HUMAN ORIGIN\*

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The relation of epidemics of streptococcal sore throat to the milk supply has directed the attention of recent workers to the pathogenic properties of the various streptococci found in milk. So far as is known all streptococci responsible for the epidemics have been of the hemolytic type, and altho an enormous amount of work has been done on milk streptococci, comparatively little has been done on the hemolytic varieties in milk, particularly in relation to pathogenesis.

By the term hemolytic streptococci is meant those streptococci which on plain-blood-agar plates cause a zone of hemolysis about the colony. This zone is usually quite clear, and varies from 1 to 3 mm. in diameter. The organisms causing atypical greenish brownish zones, or those which are very feebly hemolytic, as *Streptococcus viridans* or the pneumococcus at times may be, especially when the blood is very dilute, are not included in this group. Furthermore, it is to be understood that the hemolytic streptococci do not constitute a single variety, or genus. Hemolysis is a property common to a number of kinds of streptococci that might differ from one another decidedly in other respects. It is variable, at least within certain limits, but nevertheless sufficiently stable to be a very useful property for many practical purposes.

Ruediger<sup>1</sup> in 1912 showed that hemolytic streptococci occur in milk. In his article he assumes that hemolytic streptococci and *Streptococcus pyogenes* are identical. He concludes from his study that *Streptococcus pyogenes* seems to occur but rarely in milk and that when it does, it is indicative of the existence of an inflamed condition of the udder of the cow. *Streptococcus lacticus*, he asserts, differs from *Streptococcus pyogenes* in that the colonies of the former are greenish, with little or no hemolysis, whereas those of the latter are surrounded by a large zone of hemolysis. He considers that *Streptococcus lacticus* has no sanitary significance whatever, while the hemolytic variety is to be looked on with suspicion. He isolated in a number of instances hemolytic strains from the diseased udders and teats of cows.

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<sup>1</sup> Science, 1912, 35, p. 223.

When Dr. Capps and I studied the milk epidemics of sore throat in Chicago in 1911 and in Jacksonville, Illinois, in 1914, we isolated from the milk direct from the diseased udder of certain cows strongly hemolytic streptococci in pure culture. They resembled the human strains isolated from sore throats. Strains of streptococci were isolated from milk from other diseased cows which were not hemolytic, and which differed in other respects from the human strains. Our results were published in a paper,<sup>2</sup> the introductory statement of which reads as follows:

"The Chicago epidemic of septic sore throat reported by Capps and Miller was traced to a contaminated milk supply at Batavia, Ill. It was found that on a number of farms supplying milk to Dairy X, sore throat occurred in the milkers and mastitis appeared in the cows; in some instances the sore throat appeared first and in other instances the mastitis preceded the throat infections. These facts bring up the question whether or not it is possible for milkers suffering from sore throat to infect the teats or udders of cows by contaminated hands or otherwise during the process of milking. The question of the susceptibility of the cow to human streptococci is also involved. In an attempt to clear up these points some experiments were designed to test the pathogenicity of human streptococci for cows and to determine possible avenues of udder infection through the teats."

It was clearly shown in our experiments that hemolytic streptococci of human origin, when introduced by catheter into the healthy udder of a cow, continue to grow and are shed for a considerable period of time, and that this is accompanied by certain evidences of mastitis. Furthermore, experiments made by swabbing human streptococci about the meatus of the healthy teat did not give rise to an ascending infection, but when the teat was injured by scarifying, the streptococci ascended the ducts and caused an infection manifested by marked increase in leukocytes, the presence of large numbers of the hemolytic streptococci, but not by any physical signs. The streptococci continued to be shed in the milk for a period of at least 4 weeks, at the end of which there were still large numbers of streptococci and leukocytes in the milk. This work has been confirmed and extended since by Dr. George Mathers (p. 222) in this number), who has studied especially the behavior and effects of streptococci of human and bovine origin when grown for a long period of time in the udder of the cow.

In a recent paper Theobald Smith and J. H. Brown<sup>3</sup> showed that streptococci which were the agents of a number of outbreaks of sore throat were "all alike in that the colonies produce immediately around them a clear hemolysed area on blood agar plates (horse blood)." They state: "Our studies extending over more than a year and a half have shown that cultures from throats affected with tonsillitis contained at least two types of streptococci well differentiated on horse blood agar plates. Our attention was largely restricted to one of these types, a streptococcus producing around the colony a clear zone three to four millimeters in diameter. This type corresponds with the hemolytic strains of earlier milk-borne epidemics of tonsillitis (Boston, Chicago, and Baltimore). Within each of these groups a close analysis of morphological characters did not bring out differences beyond slight variations in size of the cocci, but on culture media differences were evident." They divide streptococci on the basis of hemolysis into 2 types: Type A, in which the colony has a partly discolored and hemolyzed mantle between it and an outer clearer zone; and Type B, in

<sup>2</sup> Capps and Davis: *Jour. Infect. Dis.*, 1914, 15, p. 135.

<sup>3</sup> *Jour. Med. Research*, 1914, 31, p. 455.

which the colony is surrounded by a clear zone of hemolysis. The former are nearly always nonpathogenic for rabbits; the latter are commonly pathogenic for rabbits, tho many strains are not. The cow streptococci were nonpathogenic for rabbits except 2 strains of the B type and possibly 1 of the A type. Smith and Brown, in looking for a sufficient reason for these explosive epidemics of tonsillitis in addition to the possible contamination of the milk during milking or later, point to the possibility of the occasional infection of the udder ducts with human streptococci. Such a possibility would be supported by the discovery of hemolytic streptococci of the human type in the milk (Outbreak B) from a cow in the suspected herd; also by the discovery in the mixed milk in the study of another epidemic (Outbreak G) of a strain not distinguishable from human pathogenic strains. The ordinary mastitis or garget streptococci, he infers, are different from streptococci of human tonsillitis and do not cause throat infections in man. Only rarely might human types find their way into the ducts through manipulations, and continue to be shed into the milk for some time.

Smith and Brown did not test this point experimentally on cows. It is evident, however, that the assumption made by these writers as regards the possible occurrence of streptococci of the human type in udders of cows was directly tested and supported by the experimental evidence obtained by Dr. Capps and myself previously, and this is pointed out by them in their paper.

Recently Krumwiede and Valentine<sup>4</sup> reported a milk epidemic in Rockville Centre, Long Island, in which they found in one cow the wide-zone hemolytic streptococci of the human type. They present evidence which they have interpreted as showing that the epidemic was largely caused by the udder infection of the cow, but that, in conformity with the assumption here discussed, this cow had itself been infected with human streptococci from a milker who was suffering from sore throat. The cow showed no evidence of mastitis except the flocculent character of the milk from one quarter. In identifying streptococci from human and bovine sources they insist on cultural identity in every detail, or immunologic identity. They give no data as to the virulence of their strains of streptococci for animals.

Savage<sup>5</sup> several years ago carried on certain experiments which have a distinct bearing on this problem, tho not altogether comparable with our own because made with goats and mice or guinea-pigs. The determination of the streptococci was also made on a different basis, no attention being given then to their reaction on blood media. However, his work points to the existence of 2 quite distinct types of streptococci as the cause of mastitis in cows, the one virulent for rodents and avirulent for goats, the other virulent for goats and avirulent for mice. The former agree in the main with the human type of streptococci which are found in infected throats, but which only rarely are found as the cause of mastitis in cows. The second type, however, is the common cause of mastitis and this organism Savage calls *Streptococcus mastitidis*. He tested *Streptococcus mastitidis* on man by inoculating his own throat with massive doses of this culture. In 2 separate experiments no ill effects were observed. It should be pointed out that he did not test on man the other variety, but he makes this statement: "In one case, for example, a streptococcus of extremely high virulence to rodents was found to be the cause of the mastitis and this organism in many other ways was quite distinctive from the streptococcus mastitidis and may well have been potentially virulent to man. If we accept the view that the ordinary type of bovine mastitis is due to organisms

<sup>4</sup> Jour. Med. Research, 1915, 33, p. 231.

<sup>5</sup> Milk and the Public Health, 1912.

non-virulent to man, but that in certain uncommon cases this condition is caused by streptococci highly pathogenic to man, it offers a complete explanation of both the bacteriological investigation and the epidemiological facts. From a practical point of view it is well to remember that the pathogenic and non-pathogenic types of bovine mastitis are not clinically distinguishable."

I wish to point out that, tho the data presented by Savage do not permit us to compare this organism, which he mentions as possibly pathogenic to man, with the human type in respect to hemolysis and some other characteristics, it appears most probable from the facts mentioned that he encountered this type in this instance. His data therefore are in entire accord with the data already analyzed in connection with this problem of the possible existence of a human and a bovine type of streptococcus as the cause of mastitis in cows.

Stokes and Hachtel<sup>6</sup> in their work on the Baltimore epidemic isolated a streptococcus of the epidemicus type from the mixed milk from one dairy, which conformed in all essential properties with the type from the human cases of septic sore throat. Other types of organisms and also slightly virulent pneumococci were isolated from milk which was suspicious on account of the high pus content.

In a number of other epidemics of milk origin, hemolytic streptococci virulent for animals have been obtained from milk from cows suffering with mastitis and some of these strains belong to the human type, as in the epidemics reported by North, Avery and White<sup>7</sup> and by Rosenow and Moon.<sup>8</sup>

While the hemolytic type of streptococcus has thus far been the causal agent in probably all epidemics, the possibility of nonhemolytic streptococci being pathogenic for man should be kept in mind and, as Theobald Smith says, the success in tracing an epidemic to its source depends on a detailed study of individual strains of streptococci and the discovery of certain minor distinguishing characteristics as guides. All hemolytic varieties are not necessarily pathogenic for animals or for man. Neither are all nonhemolytic streptococci avirulent. But these latter are not, so far as we know, concerned in causing epidemic septic sore throat.

In this connection it may be stated that nonhemolytic or feebly hemolytic streptococci may cause a distinct and long enduring mastitis in cows. I have isolated 3 such strains in pure culture directly from the inflamed udder of as many cows. They appear in the gargety milk in long chains and in large numbers. These organisms are harmless for rabbits unless one injects large doses (2 or more blood slants); then arthritis may develop. My impression from the data in the literature and also from my own experience is that such streptococci are not infrequently the cause of mastitis, but there is no evidence at present indicating that they are dangerous to man.

<sup>6</sup> Public Health Reports, 1912, 27, p. 1923.

<sup>7</sup> Jour. Infect. Dis., 1914, 14, p. 124.

<sup>8</sup> Ibid., 1915, 17, p. 69.

Organisms of this type include many of the streptococci in milk usually designated as *Streptococcus lacticus*. Most of these cause a green discoloration in blood media, but some may be feebly hemolytic, conforming in this regard with Smith and Brown's Type A; others cause no appreciable alteration of the surrounding media. These organisms likewise are relatively avirulent and, so far as is now known, possess no sanitary significance.

In view of the facts just stated it was thought desirable to study further the hemolysing streptococci from samples of milk obtained under various conditions, including both pasteurized and certified milk. From such milk a collection of strains was isolated and subjected to various tests, and was also compared in different ways with a collection of human hemolytic streptococci, particularly in regard to their pathogenicity for animals. Special attention was given to the study of the property of heat resistance on account of its relation to pasteurization. Only those streptococci were selected the colonies of which were surrounded by a distinct clear zone of hemolysis on human-blood-agar plates. (Type B, Theobald Smith.) The feebly hemolytic streptococci (Type A) were often noted in the milk, but were disregarded, since the interest in sore-throat epidemics has centered about the cocci with a clear wide zone. Other bacteria, both cocci and bacilli, were met which gave hemolytic colonies on blood plates similar to streptococci, but these were carefully excluded by suitable tests. *Streptococcus mucosus* was not encountered.

Three hundred twenty-eight specimens of bottled milk were collected from 9 different dairies in the city of Chicago. In Table 1 is given a summary of the results of the examinations made. All the specimens were pasteurized except the samples from Dairy 1, which furnished certified milk; and with the exception of the samples from Dairies D and E the holding process was used.

These examinations were made from October 1914 to March 1915. During the winter months the bacterial count on the whole was lower. The examinations were made shortly after the samples of milk had been delivered at the laboratory. Sometimes the bottles were kept in an ice box for a few hours.

Blood agar (human) was used in planting and the counts were made after incubation at 37 C. for 48 hours. The colonies of hemolytic streptococci were carefully noted and counted on the plates and later their identity was confirmed by further tests.

The lowest total bacterial counts occurred in the certified milk, the average of 45 samples being 12,306. Milk from certain of the dairies ran consistently high in bacterial counts (G and H); that from others consistently low (B).

Eighty-five samples yielded on culture streptococci of the strongly hemolytic variety. From Table 1 it is seen that the number in different samples varies considerably, ranging from a few hundred to several thousand to the cubic centimeter. The certified and the pasteurized samples contained about equal

numbers. In 16 specimens of milk from Dairy B no hemolysing streptococci were found, but this was the only exception.

A study of the 85 strains of hemolysing streptococci was made as regards their morphology, cultural characteristics, and certain other properties.

The shape of these cocci varies; it is often spherical, but frequently is more or less elongated; some strains have the stockade appearance. Individual strains under different conditions and on the various media vary somewhat.

The arrangement in milk is usually in the form of a diplococcus; short chains of 3 or more are however very common. The arrangement depends largely on the medium. At times long chains may form, especially in broth. This feature is of little value in classifying the organisms.

TABLE 1  
HEMOLYTIC STREPTOCOCCI IN MILK FROM VARIOUS DAIRIES

Dairy	Number of Samples	Range of Bacteria per c.c.	Average Number Bacteria per c.c.	Samples Containing Hemolytic Streptococci		Range of Hemolytic Streptococci per c.c.	Average Number Streptococci per c.c.
				Number	Percentage		
A	77	6,000 125,000 4,600	30,416	5	6.4	200 600	418
B	16	29,200 12,000	13,096	0	0.0	0	0
C	41	90,000 17,600	43,400	10	24.4	200 8,000	2,375
D	8	120,000 18,000	55,449	1	12.0	500	500
E	9	60,000 11,000	31,220	2	22.0	300 500	400
F	22	51,000 36,000	26,131	6	27.2	250 600	408
G	11	240,000 16,700	129,300	5	45.4	1,500 8,000	4,400
H	99	168,000 6,900	76,462	38	38.3	200 18,000	2,690
I*	45	240,000	12,306	18	40.0	200 3,000	1,028
Total	328	.....	.....	85			

\* Certified.

They are in general distinctly gram-positive, tho a few strains at times stain irregularly by this method.

In plain broth their growth is variable. The turbidity is usually distinct. In many strains a fairly abundant sediment settles out with clearing of the medium, the sediment often adhering to the sides of the tube. They grow more profusely on the whole in this medium than do ordinary human strains of *Streptococcus pyogenes*. The addition of serum to broth increases the growth.

All the cultures when inoculated into litmus-milk tubes multiply at a temperature of 20 C. The milk is slowly acidified and later coagulated. In 24 hours many strains have turned the milk pink at this temperature; other strains grow

more slowly but at the end of 4 or 5 days all the tubes have turned. At 37 C. the milk is rapidly acidified and coagulated; in the lower part of the tube often the milk is pale pink or nearly white, while near the surface it is a deeper shade of red.

Of 79 strains of these streptococci tested on 8 sugars, all fermented dextrose, lactose and maltose, 11 failed to ferment saccharose, 65 failed to ferment mannite, 11 failed to ferment salicin, 3 fermented inulin, and 5 fermented raffinose.

Titration data of 14 milk strains on various carbohydrate media are given in Table 2. The figures represent the percentages of normal acidity developed in 1% sugar broth in 1 week at 37 C. The first 10 strains in the table are the milk strains described in the foregoing. The last 4 are virulent hemolytic streptococci, 3 of which were isolated from human lesions and 1 (No. 6) was isolated from the udder of a cow.

A study of hemolysis was made both on the original plates, and on plates subsequently made. The zone is usually clear, and the corpuscles immediately beneath and around the coloring are disintegrated. The diameter of the hemo-

TABLE 2  
ACTION OF MILK STRAINS AND HUMAN STRAINS ON CARBOHYDRATES

Strains of Streptococcus	Glucose	Lactose	Maltose	Saccharose	Salicin	Mannite	Raffinose	Inulin
72	6.05	4.37	5.05	1.1	6.05	3.35	1.0	0.95
183	6.15	5.05	5.05	0.9	5.05	1.00	1.0	0.95
300	6.25	4.55	5.55	1.5	5.05	1.0	1.1	1.25
134	6.37	4.55	4.05	4.6	4.55	0.98	0.85	1.08
290	5.05	4.65	3.35	5.05	5.05	0.95	0.83	1.20
41	6.55	3.65	5.25	4.25	4.05	0.95	1.00	1.0
140	5.87	4.55	5.05	3.55	4.85	3.05	1.00	0.91
187	5.71	5.79	4.95	2.55	4.71	1.00	0.95	1.0
228	6.35	2.71	5.25	4.65	4.71	1.5	1.5	1.0
310	6.05	4.87	5.05	5.55	5.35	0.9	1.2	0.91
208*	6.25	4.71	3.85	3.55	4.55	0.9	1.1	1.0
211*	4.55	3.65	3.85	3.55	4.35	0.9	1.0	1.08
217*	4.70	3.60	5.10	5.70	6.50	1.0	1.0	1.0
6†	6.10	4.60	5.30	4.70	5.10	1.0	0.9	1.08

\* From human cases.

† Virulent strain from bovine mastitis.

lytic circle varies, from 1 to 4 or more millimeters. It may be grayish or slightly turbid. The margin of the hemolytic zone is fairly sharp, tho an occasional exception shows a margin shading off gradually into the surrounding blood. Such zones are as a rule much larger than the ordinary type. According to the description and classification of Smith and Brown this type of hemolysis would correspond to that observed with their Type B.

Tho all these strains belong in the hemolytic group the degree and character of hemolysis in all instances may not be uniform. The strains, on the basis of hemolysis, have arbitrarily been arranged in 4 groups as follows: 1st, wide clear hemolytic circle, from 2 to 4 or more mm. in diameter; 2nd, clear hemolytic circle from 1 to 3 mm. across, with diffuse and indefinite margin; 3rd, small clear hemolytic

circle from 1 to 2 mm. across with sharp margin; 4th, gray-green or hazy hemolytic circle from 1 to 1.5 mm. across, hemolysis often incomplete in circle. The 1st group passes more or less gradually into the 2nd group; the 2nd into the 3rd, and the 3rd into the 4th. This grouping demonstrates that there is a more or less gradual transition from 1 type to the other, and indicates, as was observed, that a strain may change to some extent its type of hemolysis. After carrying these cultures through many generations for a period of 9 months changes from one type to another were not uncommon, as for instance from the 3rd to the 4th; but a clear-cut transition from the clear hemolytic type to a strictly nonhemolytic or a feebly hemolytic organism was not observed. On the whole, they preserve their individual characteristics with considerable, tho not absolute, regularity. In the animal experiments to be detailed transitions from the hemolytic to a strictly nonhemolytic type were likewise not observed, tho slight changes in the type of hemolysis did occur. These observations<sup>9</sup> on the whole are in accord with my previous observations on the hemolytic properties of pathogenic streptococci. They also coincide with the notations of Smith and Brown on this point: "The hemolytic activity of the B types has remained fairly constant. In no case has it disappeared. Strain B-15 from a cow forms an apparent exception, but this strain has in the course of our studies split up into a series of forms differing in their laking capacity, some being nonhemolytic at present."

A study has been made of this group of streptococci as to their ability to resist heat. In connection with our work on the Chicago and Batavia milk epidemics<sup>10</sup> in 1912 I tested the heat resistance of a number of the strains isolated from human sources, and also of one from a suspicious cow with mastitis. These strains all were readily killed at 60 C. after an exposure of 30 minutes. This test was made in order to determine whether or not they could resist the process of pasteurization. Their resistance to lower temperatures was not determined at that time.

Since then the work of Ayer and Johnson<sup>11</sup> on this subject has appeared and is important in this connection. These writers show that a wide variation occurs in the thermal death point of strains of streptococci under conditions similar to pasteurization. For example, 33.07% of their 139 strains of streptococci were able to survive pasteurization temperature (145 F) for 30 minutes; 2.58% were able to withstand 71.1 C. (160 F.), and all were killed only at 73.9 C. (165 F.). They roughly classify the streptococci according to chain-formation into typical and atypical organisms. The latter were decidedly more resistant to heat than the former, only 1 of 22 strains of the typical class resisting a temperature of 62.8 C. (145 F.). The properties of pathogenesis and

<sup>9</sup> Davis: Jour. Infect. Dis., 1913, 12, p. 386.

<sup>10</sup> Davis: Jour. Am. Med. Assn., 1912, 27, p. 1852.

<sup>11</sup> Jour. Agr. Research, 1914, 2, p. 321.

hemolysis were not correlated with the heat resistance in their study. These organisms were isolated from the mouth, feces, and udder of the cow and from milk and cream.

In determining the thermal death point of these streptococci I used practically the same technic as that used by Ayer and Johnson, as follows: Broth cultures were grown in the incubator for from 18 to 24 hours. From each culture 4 drops in a small pipet were inoculated directly into the milk in the milk tubes. Such inoculated milk tubes were placed in a water bath held at a constant required temperature for 30 minutes. The temperature was controlled by a second standard thermometer in a tube of milk in the water bath. The tubes after heating were removed and immediately cooled to 10 C., or lower, in water; then incubated at 37 C. and observed from day to day for several days for growth.

In this manner the thermal death point of 74 strains of the hemolytic streptococci isolated from milk was determined, as summarized in Table 3. In this table the heat resistance of 24 strains of pathogenic hemolytic streptococci which

TABLE 3  
TEMPERATURE RESISTANCE OF MILK STREPTOCOCCI AND PATHOGENIC STREPTOCOCCI

Temperature, 30 Minutes		74 Strains of Hemolytic Milk Streptococci		24 Strains of Hemolytic Pathogenic Streptococci	
		Number of Strains Alive	Number of Strains Killed	Number of Strains Alive	Number of Strains Killed
115 F.	46.1 C.	74	0	24	0
120 F.	48.9 C.	74	0	24	0
125 F.	51.7 C.	74	0	10	10
130 F.	54.5 C.	74	0	10	14
135 F.	57.2 C.	74	0	2	22
140 F.	60 C.	74	0	0	24
145 F.	62.8 C.	71	3	0	24
150 F.	65.6 C.	53	21	0	24
155 F.	68.3 C.	20	54	0	24
160 F.	71.1 C.	0	74	0	24
165 F.	73.9 C.	0	74	0	24

I have isolated from various lesions and whose pathogenicity has been determined on rabbits is also presented. These were all of human origin, except 2 strains from diseased udders of cows, one isolated during the Chicago epidemic of sore throat in 1911, the other in 1914 in the epidemic at Jacksonville, Illinois. These organisms were quite like the strains isolated from human lesions of various kinds and they find their place in this group. They were pathogenic for rabbits. Five strains were isolated from human cases, either from throats or from peritoneal fluid at autopsy during the Chicago and Jacksonville epidemics.

From Table 3 it is evident that the pathogenic streptococci are decidedly less resistant to heat than the milk streptococci, the thermal death point of the former ranging from 48.9 to 60 C. (120 to 140 F.) and the latter from 60 to 71.1 C. (140 to 160 F.). This reaction is strikingly definite. The milk streptococci here tested were obtained from pasteurized milk, except the 18 strains isolated from certified milk. One would expect the process of pasteurization to have a selective action in killing off the less resistant strains of hemolytic streptococci. The results in Table 3 show that this does not seem to have been true. In 40% of the certified samples the hemolytic cocci were found; in only 1 other dairy was this percentage surpassed (Dairy G. 45.4%). The average number

of streptococci to the cubic centimeter of milk in those certified samples which contained them occupied an intermediate position in relation to the pasteurized samples. A comparison of the number of hemolytic streptococci from the same milk before and that after pasteurization, tho desirable, could not be undertaken during this investigation.

These results, therefore, should not be interpreted as indicating the heat resistance of all the streptococci that might come directly from the udder of the cow and from the usual sources of milk contamination. The results with the certified milk should afford such data, but the number of such strains (18) is small on which to base a general conclusion.

The thermal death point of the human strains was tested carefully at 2 different times during an interval of 9 months. No important differences were noted between the two series of observations. From the data in the table the necessity of efficient pasteurization is self-evident.

I have gained the impression from many observations that the virulence for rabbits of a given strain of hemolytic streptococci runs rather parallel with its virulence for man. Hemolytic streptococci highly virulent for men are highly virulent for rabbits; when slightly virulent for men they have little virulence for rabbits. In my own work on streptococcal infections in general these animals have proved themselves invaluable and highly superior to guinea-pigs for the determination of virulence. The importance of this test in the study of streptococci from milk and from bovine sources is striking. Rabbits were therefore used regularly in this work and all strains as soon as possible after isolation, were inoculated intravenously.

Streptococci vary markedly in their virulence. The ordinary hemolytic *Streptococcus pyogenes* is highly virulent and the growth from one 24-hour small blood-agar slant usually makes the animal very ill and often kills in a few days. With many strains  $\frac{1}{2}$  tube or even  $\frac{1}{3}$  or  $\frac{1}{4}$  of a tube will cause acute arthritis. I have observed some strongly hemolytic streptococci which when inoculated in doses of 2 slant tubes would not kill or produce lesions in a rabbit. Such cocci are exceptional coming from human lesions. It was therefore decided in testing virulence to inject routinely two 24-hour blood-agar slants into young rabbits weighing from 800 to 1,000 gm. Strains which in this dosage did not affect such animals were considered avirulent. This is an arbitrary method of determining virulence, but some standard must be adopted. Smith and Brown in their work used 1 c.c. of a 24-hour broth culture injected into the ear vein of rabbits. This, as they correctly state, is a much smaller dose than is usually employed for such purposes. I have not been able to use successfully plain broth cultures because so many of the pathogenic streptococci grow poorly in this medium. Consequently when not using slant cultures I have used serum broth (1:5), which yields a good growth with all strains.

It is perhaps not quite right to compare broth cultures and slant cultures but estimation that the growth from 1 small blood slant is equivalent to 8 c.c. of a serum-broth culture makes our doses of 2 slants equivalent to 4 c.c. of a serum-broth culture.

Each of 85 strains of the hemolytic cocci were thus injected into the ear vein of a young rabbit of stated weight. The animals were permitted to live 10 days; they were then killed and examined. Cultures were made in all cases from the heart blood, and in most cases from the gallbladder; also from any suspicious lesion, especially joint lesions.

With the dosage as given 15 strains produced lesions visible at autopsy. These involved joints and on the whole were mild. Positive cultures from joints were obtained in all cases save one. Two animals died, one on the 7th, the other on the 9th day after inoculation and streptococci were isolated from the elbow in one and from the wrist in the other. The heart blood was negative in both.

The virulence of each of these 15 strains after the 1st rabbit passage was tested still further. Two cubic centimeters of a 24-hour serum-broth culture of each were injected into the ear vein of rabbits. Eleven animals killed after 10 days showed no lesions. One died in 48 hours and streptococci were grown from the heart blood. The 3 remaining animals, killed after 10 days, revealed in 2 cases pus in the left wrist from which in each case streptococci were grown pure; in the third rabbit, pus was found in the right knee with positive culture. These 4 strains after the two animal passages were again injected in doses of 1 c.c. of a 24-hour serum-broth culture into rabbits. With this dosage no results were obtained.

From these results it is evident that all the strains in this series are relatively avirulent. None could be classed in this respect with the human types of hemolytic streptococci as found in sore-throat epidemics. It is to be noted, however, that when large doses are used there is variation in their pathogenicity, some of the strains approaching but not attaining the standard we have arbitrarily set for an organism's being called virulent. If this be true, might not milk or bovine strains, occasionally at least, attain still higher degrees of virulence for rabbits and in this respect fall in the human group?

A comparison of the 84 strains of hemolytic streptococci from milk and 24 of hemolytic streptococci from human sources was here made, the chief points of which are presented in Table 4. It should be noted that the statements made in the table under the various properties are not in every regard absolute. In the large series of streptococci of milk origin there are some which in one or more points might be properly classed with the human type, and vice versa. Each strain must be studied by itself.

The human virulent streptococci when grown in milk are more spherical, while the milk streptococci tend to be more elongated, but

since many exceptions appear, this characteristic has little value for determination purposes. This is true also of the arrangement of the streptococci in milk. The human strains commonly form long chains, while the milk strains appear in diplococci and short chains. Changing the media will often alter this characteristic markedly.

Capsules are present at times in the virulent human strain, as was particularly true of some of the epidemic strains. They may be lost under artificial conditions and acquired again by animal passage. The capsule appears to be strikingly persistent in some strains. None of the milk strains possessed a capsule. In this series the encapsulated streptococci were always highly virulent for rabbits. However, hemolytic streptococci of the human type without capsules may also be highly virulent for rabbits.

TABLE 4  
COMPARISON OF HEMOLYTIC HUMAN AND MILK STREPTOCOCCI

Characteristics Studied	Human Type	Milk Strains
Shape.....	Spherical or slightly elongated....	More elongated
Arrangement.....	Marked chain-formation .....	Diplococci or short chains
Capsules.....	May or may not be present.....	Not present
Growth in plain broth	Often not very abundant.....	More abundant
Growth at 20 C. in milk	Very slow or not at all.....	Good growth as a rule
Growth at 37 C. in milk	More slowly acidified, with, or often without, coagulation	Rapidly acidified and coagulated
Acidity in sugar broths	Moderate .....	Higher
Hemolysis.....	Zone wide and usually clear.....	Zone narrower and at times turbid (many exceptions)
Heat resistance, 30 minutes	120-140 F. (48.9-60 C.).....	140-160 F. (60-71.1 C.)
Pathogenicity for rabbits	Marked .....	Very little or none
Pathogenicity for cow	Marked .....	Very little

In plain broth, growth of many human strains is not abundant, though there are notable exceptions. The milk strains grow more vigorously and profusely in this medium. At 20 C. in milk the growth of the human strains is slight or at a stand-still, while the milk strains grow well. At 37 C. in milk the human strains acidify with or without coagulation; the milk strains acidify more rapidly and coagulate in every instance, the medium usually being pink at the top and white beneath.

In the fermentable carbohydrate broths the milk strains grow more profusely, acidify more quickly, with generally a higher terminal acidity.

Comparing the 2 groups with reference to hemolysing property we find no really important or constant differences. Those from human sources possess a somewhat wider and clearer zone than those from the milk, but there are so many exceptions that such a statement has little value. Just as there are slight differences, already noted, between milk strains in the character of the hemolytic zone, so there exist similar differences between many of the human strains. Such have been referred to in my earlier papers,<sup>12</sup> when I was studying the properties of the epidemic streptococci. Between hemolytic area and

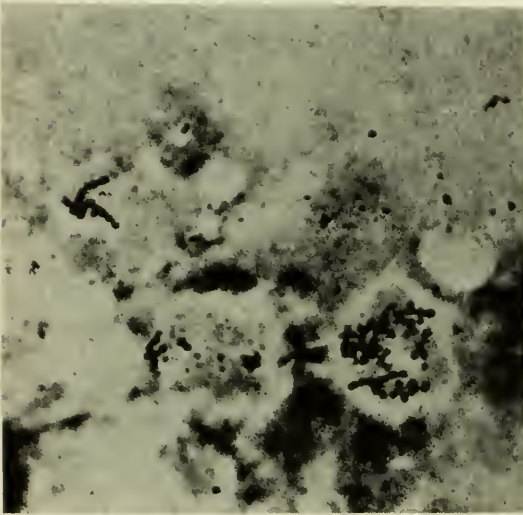


Figure 1

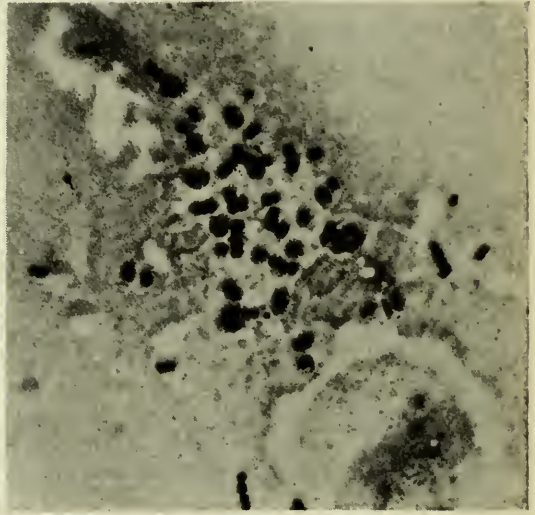


Figure 2

Fig. 1. Hemolytic streptococci stained directly in milk from cow suffering with mastitis (5th week). Phagocytosis is evident. Capsules very doubtful.

Fig. 2. Same strain as shown in Fig. 1. In the body of the rabbit for which it was virulent it acquired the distinct capsules shown.

pathogenicity there exists no consistent parallelism. Indeed, it may happen that as the virulence is raised the zone of hemolysis becomes narrower. This may be due to the production of a less diffusible hemolysis, which need not necessarily be less toxic. But there is no good reason to think that the symptoms of streptococcal infections are to any appreciable degree dependent on the production of a streptolysin.

As regards heat resistance the two series have already been compared. It may be stated again that the human pathogens appear to be decidedly more sensitive to heat.

<sup>12</sup> Jour. Am. Med. Assn., 1912, 58, p. 1852.

I would repeat also that pathogenicity for rabbits seems to be a most valuable character for determining the sanitary significance of a streptococcus from human or bovine sources. There is no evidence at present to lead one to believe that a hemolytic streptococcus that is avirulent for rabbits has any pathogenic properties for man. On the other hand, all the dangerous streptococci isolated from the epidemics of sore throat are highly virulent for rabbits. The importance of the virulence for rabbits, therefore, in connection with studies of these milk epidemics cannot be over emphasized.

One of these hemolytic milk strains was injected by Dr. Mathers<sup>13</sup> into the udder of a cow, 2 c.c. of a 24-hour milk culture being used. A mild inflammation resulted; the streptococci increased in number for 2 days, then rapidly decreased and after 15 days the milk was again sterile. The number of leukocytes in general paralleled the number of streptococci; on the second day after injection they were most numerous, numbering at that time 6,200,000 to the cubic centimeter, and on the 10th day they had reached normal (43,000). A careful comparison of the behaviors of the different varieties of streptococci when injected into the udder was made by Mathers. It suffices here to state that hemolytic streptococci of the human type, coming directly either from human lesions or from a diseased udder, are decidedly more virulent for the cow than are the hemolytic milk strains.

This comparison leads us to ask whether or not we are justified in making a clear-cut distinction between the bovine type and the human type of hemolytic streptococci. Brown and Smith noted that their bovine strains had certain characteristics which distinguished them from the strains coming from human sources, with the exception of 2 strains from cows which conformed with the human type. The chief points that they make in their distinction are as follows: The bovine strains are not pathogenic for rabbits; they produce a higher degree of acidity than the human strain; in milk they produce a firm coagulum in 3 days, whereas in the case of the human strains the milk remains fluid or only a slight coagulum forms at the bottom of the tube. In these respects our milk strains agree very well with Smith and Brown's bovine strains, and probably belong to the same general group. The 85 milk strains tested may be assumed to be of bovine origin, and in contrast to the pathogenic human types, may be referred to as the bovine type of hemolytic streptococci. Most of them probably originated from the feces, skin, hair, mouth, etc., of the cow, some possibly directly from the udder.

<sup>13</sup> Jour. Infect. Dis., 1916, 19, p. 222.

The relationship of these hemolytic milk strains to the *Streptococcus-lacticus* type is apparently very close. The latter are commonly nonhemolytic or very feebly hemolytic, and often produce a green colony and a green zone on blood plates. Many strains, however, form small gray colonies without any appreciable alteration of the blood. Exclusive of hemolysis the two types are practically identical. They are both avirulent for rabbits and, as stated previously, Mathers has shown that when injected into the udder of a cow they both produce a similar mild type of mastitis, which clears up in the course of from 1 to 2 weeks.

Of the 85 strains isolated from the milk none had properties which would justify one in considering it of human origin. It is quite probable, however, that if a great number of hemolytic strains were isolated from mixed unpasteurized market milk the human type, which could be definitely identified, would be found. Such a strain might originate directly from man or from a cow suffering with mastitis caused by streptococci of the human type.

The question naturally arises as to whether or not all epidemics of sore throat are caused by streptococci from human sources that gain entrance into the udder and grow there. That this is possible is clear from our earlier experimental work and that it may actually occur is supported by the work of Smith and Brown and others. The data at hand are not, however, sufficient to exclude the possibility of bovine streptococci attaining virulence for man. Man may be susceptible to both bovine and human streptococci just as he is to both bovine and human tubercle bacilli. This possibility should not be lost sight of. The data presented in this paper indicate different degrees of virulence for rabbits on the part of the milk streptococci.

Milk containing virulent hemolytic streptococci of the human type need not necessarily be dangerous to use, tho of course such milk should be condemned. Clinical experience is against the view that in man streptococci from skin and many other lesions are always or even commonly concerned in causing throat infections. It may happen that the udder becomes infected, for example, from a streptococcal infection of the finger of a milker. Such streptococci, tho virulent, might not be able to cause an epidemic of sore throat. There is here involved the question of specific-tissue affinity, a problem that Rose-now<sup>14</sup> has recently discussed.

<sup>14</sup> Jour. Am. Med. Assn., 1915, 65, p. 1687.

The question of variability and mutation and its bearing on the data here presented is a large and difficult subject, and the experiments and observations in this paper were not designed to bear directly on this problem. It may be said that during the time and under the various conditions that the different strains of streptococci were observed, only slight variations in certain properties, as for example changes in sugar reactions, were occasionally noted. No radical change was observed in the character of hemolysis on human-blood-agar plates in any of the strains. I have, however, observed slight loss or gain in hemolytic power in some of the strains, in others slight alterations in the character of hemolysis, as has already been pointed out. That virulence of streptococci can be altered by repeated transfer from animal to animal, or by growth on artificial media, is a well known phenomenon. The variations observed in the study of these streptococci were those commonly seen by bacteriologists in their daily work in the laboratory in connection with different varieties of bacteria. Any alterations suggesting the origin of mutants were not observed. I have not obtained the impression from this or previous study on streptococci that fundamental variations or mutations play a practical rôle in problems of the kind here considered.

In regard to the variation of minor cultural details of strains, it was noted in studying streptococci during the Chicago epidemic, that in body fluids particularly the organisms tended to develop different properties from those of many cocci from the throat of the same individual. I quote<sup>15</sup> as follows:

"On sugars, milk and other mediums these two varieties grow practically alike. The relation of these two types is undoubtedly a very close one and certain facts would seem to indicate that the one is simply a modified form of the other. When grown on ordinary mediums the encapsulated coccus—at least some strains—loses its capsule and the growth on slants is less profuse and moist. By animal passage the capsule and other properties mentioned return. The profuse moist appearance of the colonies, it may be stated, seems to be dependent on the capsular substance. While this seems to be true of some strains other strains maintain their peculiar characteristics for a long time (several months) with little or no change."

In this connection Smith and Brown<sup>3</sup> say regarding their streptococci that "spontaneous changes in cultural characters do not proceed rapidly enough if they go on at all to interfere with current bacteriologic methods."

<sup>15</sup> Davis: Jour. Am. Med. Assn., 1912, 58, p. 1852.

## SUMMARY

Hemolytic streptococci having a wide clear zone occur commonly in both pasteurized and unpasteurized (certified) milk. These strains vary among themselves. They are more resistant to heat than human strains of hemolytic streptococci, and possess little or no virulence for rabbits, therefore in all probability none for man. They rapidly acidify and coagulate milk and grow well at 20 C. They form short or long chains, but as seen in milk often appear in pairs or a chain of few elements. While they are definitely hemolytic (Type B. Smith and Brown), the characteristics of the hemolytic zone on plates may vary in certain respects.

The milk strains are different from certain strains of hemolytic streptococci found at times in diseased udders in cows. These latter resemble the strains of hemolytic streptococci from human sources, and are virulent for rabbits.

There is no reason to consider that these organisms have any sanitary significance. The importance, however, of certain types of hemolytic streptococci in relation to epidemics of sore throat makes it necessary to study carefully all such organisms in milk.

By itself the hemolytic property has no more value for identification purposes than many other characteristics, but is greatly important on account of the practical value of the blood-agar-plate method as a means of initial separation of human type strains from the many strains of nonhemolytic and feebly hemolytic streptococci found in milk.

# CERTAIN NONSPECIFIC REACTIONS OBTAINED WITH ANTIGENS MADE FROM BACTERIA GROWN ON SERUM MEDIA \*

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During our work with rabbit immune sera developed against bacteria grown on serum media, such rabbit sera frequently showed a precipitate when added to a bacterial precipitogen in the preparation of which the heterologous serum had been used, but no precipitate with bacterial extracts made without the aid of serum media.

This phenomenon occurred so often that we were compelled to consider it of the greatest importance in all serologic studies depending on immune serum developed by the use of such antigens. We have been unable to find any direct reference to this factor's disturbing, as it may, comparative studies of organisms by agglutination, complement-fixation, and precipitation reactions of their immune sera.

It has already been inferred that precipitation is a process analogous to agglutination, and the results of our investigations add support to such an assertion. Not alone this, but complement-fixation seems to compare with these in the curve of its development.

Our object is to show the simultaneous development of nonspecific agglutinins, precipitins, and complement-fixing bodies by the injection of bacteria grown on serum agar.

## THE ACTION OF RABBIT VERSUS HUMAN PRECIPITATING SERA

Since human ascitic fluid is the usual source of the serum employed in making the serum media for bacterial growth, our first endeavor was to immunize rabbits against such fluid.

We have tried several methods of producing precipitating serum. The intensive one advocated by Hektoen for blood serum produced no appreciable precipitin with ascitic fluid. In our experience the older method of increasing the amounts of antigen over a long period of time produced the most satisfactory results, as is illustrated in the following case:

\* Received for publication February 14, 1916. Work done in part under tenure of the Eugene Meyer Jr. and George Blumenthal Jr. fellowships in pathology.

*Rabbit 4.*—Injected with 5, 10, 15, and 20 c.c. of human ascitic fluid at 4-day intervals, the first two having been given intravenously, the last two intraperitoneally, yielded 10 days after the last injection a serum the precipitin titer of which was 1:1000 (read at the end of one hour at 40 C.).

The slow production of precipitin in the case of ascitic fluid is important, the bacterial antigens employed by us requiring at least 4 injections, more often 7 or 8, before a satisfactory immune serum was produced.

In studying the serologic properties of rabbit precipitating serum, especially in relation to organisms grown on serum media, we noted that while normal rabbit serum showed no precipitin, agglutinin, or complement-fixing bodies against such bacteria, this serum showed such reactions to a considerable degree. Where the precipitin titer was lower, the reactions were correspondingly weaker, and when, in spite of injection of antigen, there was no precipitin response, such rabbit serum gave no similar serologic effects. In the technic of the tests to determine these factors, ample controls were always used to allow for the variations in normal rabbit serum in regard to its antibody content. The serum was tested before the rabbit was employed experimentally and all reagents were carefully titrated in the tests.

Applying these principles, we compared precipitating (rabbit versus human) serum with rabbit immune serum produced by the repeated injections of organisms grown on human-ascitic-agar media.

#### TECHNIC

For this purpose a number of bacteria were employed, mostly of the diphtheroid group, but of different cultural characteristics. All were grown on serum agar for a greater or lesser period of time (see Table 1).

TABLE 1  
THE CHARACTERISTICS OF THE ORGANISMS EMPLOYED

Organism	Description	Source	Length of Time on Serum Media Before Use
7	Gram-positive anaerobic bacillus.....	Acne pustule.....	5 months
c	Gram-positive anaerobic bacillus.....	Ascitic fluid.....	8 months
3*	Gram-positive anaerobic bacillus.....	Acne pustule.....	9 months
III	Same as 3 but grown aerobically.....	Acne pustule.....	9 months
96	Gram-positive anaerobic bacillus.....	Ascitic fluid.....	8 months
9	Gram-positive anaerobic bacillus.....	Acne pustule.....	2 months
R9	<i>Streptococcus viridans</i> .....	Tonsillitis.....	3 years
464	Gram-positive aerobic streptobacillus.....	Urine.....	5 months
332	Gram-positive aerobic streptobacillus.....	Urine.....	7 months
SA	<i>Staphylococcus aureus</i> .....	Blood.....	Varying in time
Pn	<i>Pneumococcus</i> .....	Sputum.....	4 days

\* Organism through the courtesy of Dr. C.-E. A. Winslow.

A series of rabbits were then immunized against Organisms 7, 3, III, and 96. These bacteria, being nonvirulent for rabbits, were injected alive, intravenously. The growths were very carefully washed off with salt solution, so that no media entered into the composition of the antigen. After 4 injections, given at 4-day intervals, consisting of the growth of 1, 2, 3, and 4 standard serum-agar slants, respectively, an interval of 10 days was allowed to elapse, and then the rabbit was bled for trial of antibody content. Usually, it was found necessary to give 2 or 3 additional injections to produce a practicable immune serum, i. e., one having an agglutination titer of about 1:400 and showing complement-fixation in 0.005 c.c. of immune serum.

*Antigens.*—Antigens for complement-fixation were prepared from the organisms by suspending the growths from several serum-agar slants in distilled water. Here, as before, care was taken in washing off the growth to avoid carrying over any of the medium into the extract. These suspensions were then allowed to autolyze at 60 C. for 1 hour and at 37 C. for 24 hours. The materials were now centrifugated until clear supernatant fluids resulted, and these were used as antigens. For the tests, they were "normalized" with 9% salt solution, to obviate their lytic activities. They were then titrated and one-quarter of the anticomplementary dose was employed. The tests were set up as in the Wassermann test except that one-half the quantities were used. In order to eliminate reactions due to anticomplementary action, not only were the rabbits tested before use with the same antigens, but at least 4 units of a hemolytic system were employed. The rabbit immune sera were inactivated, and fixation allowed for one hour at 37 C. and 3½ hours in the ice-box.

*Agglutinogens.*—Agglutinogens were prepared from these organisms by suspending growths from serum-agar slants in salt solution, with the same precautions mentioned in the foregoing to eliminate particles of media from the reagents. The suspensions were thoroughly shaken before use. Inactivated immune sera were used and the microscopic method of reading employed. It was obviously impossible to make these tests by the macroscopic method since the clumps of the precipitate formed interfered with the readings.

*Precipitin Tests.*—These tests were always made with inactivated immune sera and in serial dilutions made up with normal salt solution. At all times a normal rabbit serum was included as control.

*Serum Media.*—The media consisted of 0.5% glucose 2% agar slants, which were made up with one-third to one-quarter the volume of fresh ascitic fluid. The acidity of the media varied between 0.9 and 1 acid to phenolphthalein.

Having produced a rabbit precipitating serum by injection of ascitic fluid, we now applied it to the serum-grown organism for the purpose of studying its action as regards complement-fixation and agglutination, following the technic just described.

As for complement fixation, the following results were obtained. There was no fixation with normal rabbit serum when Antigens 96, 9, 3, 7, and C were used. In the case of the serum from Rabbit III, which produced a precipitin against ascitic fluid only in a dilution of 1:50, the fixation with Antigens C and 9 using 0.01 c.c. rabbit serum

was complete, +++ with Antigen 7, ++ with Antigen 3, and negative with Antigen 96. Comparing this with the serum from Rabbit 4, which produced a precipitin active 1:1000 against ascitic fluid, we note a correspondingly stronger fixation with all these antigens. For comparison see Table 2.

TABLE 2  
THE NONSPECIFIC COMPLEMENT-FIXATION TESTS WITH PRECIPITATING SERUM

Antigens	Normal Rabbit No Precipitation			Rabbit 3, 1:50 Precipi- tation Titer			Rabbit 4, 1:1000 Precipi- tation Titer		
	0.05 c.c.	0.01 c.c.	0.005 c.c.	0.05* c.c.	0.01 c.c.	0.005 c.c.	0.05 c.c.	0.01 c.c.	0.005 c.c.
3	0	0	0	++++	++	0	++++	++++	0
96	0	0	0	++++	0	0	++++	++	0
C	0	0	0	++++	++++	0	++++	++++	++
9	0	0	0	++++	++++	0	++++	++++	+++
7	0	0	0	++++	+++	0	++++	++++	0

\* Serum slightly anticomplementary in this amount.

In regard to agglutination, a similar curve of reaction between a precipitating (rabbit versus human) serum, and the various serum-grown organisms, was observed (see Table 3). We note from these tables the co-relationship which exists between the elaboration of complement-fixing bodies and that of agglutinating bodies in a precipitating nonspecific serum. On extending our investigations, as far as aggluti-

TABLE 3  
THE NONSPECIFIC AGGLUTINATION TESTS WITH PRECIPITATING SERUM

Agglutinogens	Normal Rabbit No Precipitation			Rabbit 3, 1:50 Precipi- tation Titer			Rabbit 4, 1:1000 Precipi- tation Titer		
	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200
Serum dilutions									
3	0	0	0	0	0	0	++	++	+
96	0	0	0	+	+	+	++	++	+
C	0	0	0	+	+	+	++	++	+
9	0	0	0	+	+	0	++	++	+
7	0	0	0	0	0	0	++	++	++
Control (non- serum organ- ism).....	0	0	0	0	0	0	0	0	0

nation is concerned, to other organisms of various types that have been grown on serum media for a considerable time (see Table 1), we obtained the following results:

In the case of *Streptococcus viridans* (R9) grown for over 3 years on serum agar, agglutination—all positive agglutination titers varying between 1:200 to 1:400—was equally strong, not only with precipitating and normal rabbit serum, but also with human ascitic fluid. This

effect of the ascitic fluid's influencing agglutination, which cannot as yet be explained, will be the subject of future investigation. For not only in the case of the streptococcus, but also with *Staphylococcus aureus*, and typhoid bacilli, strong agglutination could be obtained. Indeed, after the 3rd transplanting to serum media of *Staphylococcus aureus*, the control itself shows considerable agglutination. This fact is not noted when the same organism is grown on plain agar for 16 generations. The precipitating serum would not agglutinate staphylococci grown on serum media up to a certain generation, after which the reaction became progressively stronger, but eventually the agglutinating power was lost. This phase will be discussed in a future communication.

The precipitating serum was found to agglutinate in this same non-specific manner Strain 464, a streptobacillus isolated from the urine and grown on serum media for 5 months. A culturally identical organism (332), which had been grown for 7 months on serum agar, was not agglutinated, neither was a pneumococcus which had been growing on such media for 4 days. In explanation of this failure to agglutinate, we must bear in mind that certain organisms, culturally identical, may absorb serum at different rates and in different quantities, and thus give divergent results when the amount in the reaction influences the reading.

#### THE INFLUENCE OF SERUM IN MEDIA UPON COMPARATIVE SEROLOGIC STUDIES

We have found, in numerous attempts to produce specific immune rabbit serum against antigens made from bacteria grown on serum media, that cross-fixation and agglutination were frequently encountered, altho the organisms differed in cultural characteristics. On the other hand, since the presence of serum in the media was the only factor common to all these antigens, we were led to study the relation of ascitic fluid to such immune serum. It was found that ascitic fluid used as precipitogen gave distinct precipitates with the various sera.

The immune sera against the serum-grown bacteria were prepared in the manner stated in the foregoing. We feel certain that the great care exercised in preparing the antigens for injection excluded the possibility of carrying over particles of media on serum in itself. Hence it becomes obvious that the bacteria themselves are the carriers in some manner of the serum proteins in sufficient quantities to cause specific serum antibodies. Thus, immune sera against Organisms 3, 7,

III, 96 (see Table 2) showed precipitins using ascitic fluid as precipi-  
togen in dilutions of at least 1 : 100, whereas normal rabbits were uni-  
formly negative.

Having shown, 1st, that rabbit versus human serum precipitin is  
synchronously produced with the antibodies against these organisms,  
and 2nd, that such precipitating serum may give nonspecific agglutina-  
tion and complement-fixation, we have adequate explanation for the  
following examples of cross-agglutination and cross-fixation, noted in  
Tables 4 and 5.

TABLE 4  
NONSPECIFIC AGGLUTINATION

Immune Serum	Antigens											
	96			3			III			7		
	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400
96	++	++	+	++	++	++	++	++	++	0	0	0
3	0	0	0	++	+	+	++	++	++	++	++	+
III	0	0	0	0	0	0	++	++	++	+	+	+
7	+	+	+	+	+	+	++	++	++	++	+	+
Normal	0	0	0	0	0	0	0	0	0	0	0	0

In a study of the cellular antibodies, working with the Dale appara-  
tus, and using the methods described by R. Weil, one of us in collabora-  
tion with B. S. Denzer found that here as well, the specificity of such  
antibodies was interfered with if the antigen used for immunization of  
the guinea-pig consisted of bacteria grown on a heterologous serum  
media. For not only were cellular antibodies for the bacteria found,  
but also for the serum used in the media; so that an antigen so pre-  
pared would give marked reactions when added to a uterus immunized  
only against the homologous serum and the resulting contraction there-  
fore would in no way indicate the specificity of the bacterial antigen.  
On the other hand, we have been able to demonstrate that the same  
nonspecific factors may be transmitted passively. Thus in a rabbit

TABLE 5  
NONSPECIFIC COMPLEMENT-FIXATION

Anti- gens	Immune Sera								Normal Serum	
	96		3		III		7			
	0.01	0.005	0.01	0.005	0.01	0.005	0.01	0.005	0.01	0.005
96	++++	++++	++++	++++	++++	++++	++++	++++	—	—
3	++++	++++	++++	++++	++++	++++	++++	++++	—	—
III	++++	++++	++++	++++	++++	++++	++++	+++	—	—
7	++++	++++	++++	++++	++++	++++	++++	++++	—	—

All serum antigen controls completely hemolyzed; one-quarter of antigen anticomplemen-  
tary unit was employed. Only 2 dilutions are given to show comparisons.

immunized by the repeated intravenous injection of serum-grown organisms, an immune serum was obtained, which when injected into a guinea-pig would sensitize its uterus not only to the bacteria employed but also, with just as great response, to the serum.

By these experiments, we have shown that organisms grown on serum media incorporate in some manner those serum elements which possess serum antigenic properties. Such being the case, it follows that comparative studies of these organisms by means of immune serum would be impossible. A common factor present in the antigens foreign to the organisms themselves, leads to the formation of non-specific immune sera, that is, to antibodies against all serum-grown bacteria.

We have also shown the co-relationship between agglutinins, precipitins, and complement-fixing bodies in these rabbits. The curve of precipitin-formation is closely paralleled by that of the production of nonspecific agglutinin and complement-fixing bodies. Yet, once present, these antibodies exist as independent entities. For example, a rabbit versus human precipitating serum contains agglutinins against various serum-grown organisms. This agglutinin will persist even tho the precipitin is entirely removed.

A few experiments were conducted with some antigens which were prepared with an additional refinement in technic, in that they were filtered through a Berkefeld candle. Such antigens have shown a more specific behavior. Whether or not this method will remove all the extraneous serum elements and leave only pure bacterial protein, we are not prepared to say.

We have established the fact that organisms grown on a medium containing a heterologous serum (e. g. human ascitic medium and rabbit serum) are unsuitable as antigens for use in producing antibodies for comparative studies, and that true specific reactions can only be obtained by employing either nonserum or homologous-serum media for the growth of bacteria.

#### CONCLUSION

The injection of serum-grown bacteria into animals for the purpose of producing immune serum for comparative serologic studies is to be avoided. Such a method results in the production of a precipitating serum versus the serum present in the media. The antiserum thus formed reacts in a nonspecific manner to various bacteria grown on serum media in regard to precipitation, agglutination, complement-fixation, and formation of cellular antibody.

# BACILLEMIA IN TUBERCULOSIS AS SHOWN BY THE EXAMINATION OF POSTMORTEM CLOTS FROM THE HEART\*

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A study of the literature on the subject of the presence of tubercle bacilli in the blood of tuberculous patients reveals much conflict both in opinion and in experimental evidence. That tubercle bacilli enter the blood vessels and are generalized by means of the circulating blood is of course unquestioned; but to what extent this generalization takes place is still unsettled. Is the bacillemia in tuberculosis continuous enough or so marked that it can be demonstrated and utilized as a means of diagnosis? Particularly in acute miliary tuberculosis are the bacilli likely to be found in the circulating blood; and the demonstration of their presence there would be of practical importance in the diagnosis of early doubtful cases. Most experimental evidence indicates, however, that at no time is the bacillemia sufficient to have any diagnostic worth.

The many attempts to demonstrate tubercle bacilli in blood smears have ranged in result from the extravagant claims of Rosenberger,<sup>1</sup> since shown to rest on technical error, to the almost universally negative findings at the hands of numerous workers. Efforts to obtain the bacilli from the blood by culture or by animal inoculation have likewise given chiefly negative results. Bergeron<sup>2</sup> secured 3 positive cultures; and Faber<sup>3</sup> was successful in cultivating the organisms from blood taken at autopsy in 1 case of miliary tuberculosis. Recent investigators (Berry,<sup>4</sup> Kessel<sup>5</sup>) have also failed to demonstrate the presence of tubercle bacilli in the blood of advanced cases of pulmonary tuberculosis, either by smears, culture directly from the blood, or by animal inoculations. Much more successful has been the demonstration of tubercle bacilli in the placental blood of placentas from mothers suffering either with acute miliary or chronic pulmonary tuberculosis (Norris<sup>6</sup> and others). The not infrequent occurrence of placental tuberculosis in cases of slight latent pulmonary tuberculosis of the mother (3 cases reported from this laboratory by Warthin<sup>7</sup> and Weller<sup>8</sup>) would

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<sup>1</sup> Am. Jour. Med. Sc., 1909, 137, p. 267.

<sup>2</sup> Presse méd., 1905, 13, p. 129.

<sup>3</sup> Jour. Am. Med. Assn., 1914, 63, pp. 1656-1658.

<sup>4</sup> Jour. Infect. Dis., 1914, 14, p. 162.

<sup>5</sup> Am. Jour. Med. Sc., 1915, 150, p. 277.

<sup>6</sup> Tr. Am. Gynec. Soc., 1914, 39, p. 452.

<sup>7</sup> Jour. Am. Med. Assn., 1913, 61, p. 1951.

<sup>8</sup> Arch. Int. Med., 1916, 17, p. 509.

tend to show the occasional entrance of tubercle bacilli into the blood stream from slight and apparently latent lesions, and that in pregnant women such bacilli are more likely to fall out of the circulation into the intervillous spaces of the placenta than in any other organ.

One other method of determining the presence of tubercle bacilli in the blood after death, not yet reported in the literature on tubercle bacillemia, is the examination of the postmortem clots in the heart and great vessels by means of stained sections. This method would at least give a definite idea of the number of tubercle bacilli in the heart blood at time of death. At Dr. Warthin's suggestion I have attempted to make such an estimation on blood clots from the heart in a case of generalized miliary tuberculosis in which the dissemination was extreme and the tubercles relatively young.<sup>9</sup>

#### THE CASE

A housewife, aged 52 years, with negative family history as regards tuberculosis, became ill about Oct. 21, 1914, with a feeling of unusual fatigue, fever and slight chilly sensations, increasing toxemia, rapidly progressing anemia, weakness, and dyspnea, without any signs of localized disease. Death took place on Nov. 29.

The gross and microscopic examinations at the autopsy furnished the following pathologic diagnosis: Primary tuberculosis of bronchial nodes with secondary pyogenic infection; cold abscesses in bronchial nodes with rupture into pulmonary vein; healed tubercle in right lung; miliary focal tuberculous necroses in lungs, liver, spleen, and bone marrow; older miliary tubercles in kidney; colonies of tubercle bacilli in myocardium; multiple thromboses (tuberculous) in pulmonary veins, portal vein, and ovarian plexus; exhaustion of bone marrow; secondary anemia; gravis aplastica; fatty degeneration of heart and liver; chronic fibroid salpingitis. Tubercle bacilli in large colonies in the focal necroses.

If tubercle bacilli ever exist in the circulating blood in large numbers, this case would seem to be favorable to such a condition, because of the early stage of the tubercles, the lesion opening into a pulmonary vein, the wide dissemination of the lesions, and the apparently progressive entrance of bacilli into the circulation as shown by the varied picture of the tuberculous foci ranging from thromboses, coagulation and caseation focal necroses, to fully developed epithelioid tubercles.

The white clots and blood found in the right side of the heart were fixed in mercuric chlorid, carefully washed, and imbedded in paraffin. The sections were floated onto warm carbolfuchsin without removal of the paraffin, decolorized, and counterstained with methylene blue, washed, dried on the slide, the paraffin removed by warming and the use of xylol, and the sections mounted in balsam. These stained sections were then carefully searched for tubercle

<sup>9</sup> Marshall: Arch. Int. Med., 1915, 16, p. 1045.

bacilli. In all, about one-tenth of the entire mass of clot and blood obtained from the heart (chiefly white clots from the right auricle) was examined in this manner. Only 4 typical slender and beaded acidfast bacilli were found in the blood-clot sections. Sections of the tuberculous lesions in the organs stained in the same manner showed the presence of similar acidfast bacilli in enormous numbers in the focal necroses.

If the other nine-tenths of the heart blood and clot not examined contained tubercle bacilli in the same proportion as the one-tenth examined, then the entire number contained in the heart blood at time of death would be 40 or less. In this case, as the disease was in a most acute and severe form and the organisms were being generalized throughout the body in large numbers, the number of bacilli found in the blood at any given moment would have to be relatively small, and the chances for demonstrating their presence by stained smears, cultures, or animal inoculation could not be very favorable. In milder cases, and in cases of chronic pulmonary tuberculosis, the chances for such a diagnostic demonstration of the bacilli in the circulating blood would seem to be very small indeed.

# TUBERCLE BACILLI IN THE HEART CLOTS IN ACUTE MILIARY TUBERCULOSIS COMPLICATING CHRONIC LYMPHATIC-MYELOGENOUS LEUKEMIA \*

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It has been shown by Wilson<sup>1</sup> that tubercle bacilli can be demonstrated in stained sections of blood and clots obtained at autopsy from the heart and great vessels of a case of acute miliary tuberculosis, but that, as already borne out by the clinical attempts to demonstrate tubercle bacilli in the blood by means of stained smears, culture, and animal inoculation, the number of bacilli in the circulating blood at any given moment is too small to be of any practical diagnostic value. The wide-spread dissemination of the lesions in the case studied, occurring as they did even in the bone marrow, and the very early stage (thromboses and focal necroses) of many of the lesions would seem to make it a case in which the maximal number of bacilli might be found in the blood stream. That so few (4 bacilli in one-tenth of the heart clots) were found in the heart blood at any given moment cannot be taken as an absolute index of the number of tubercle bacilli entering the blood stream from a primary focus. Rather may it show, what we already know experimentally, that tubercle bacilli entering the blood stream quickly disappear from the circulating blood. The clots and blood in the great veins and right heart should contain few bacilli, as they have dropped out of the blood during its arterial and capillary circulation. It is possible that in an ordinary case all the tubercle bacilli entering the pulmonary veins from a primary bronchial-gland focus, and passing into the arterial blood, might disappear from the venous blood by the time it reached the right heart. That this did not take place in the case described is demonstrated by the presence of bacilli in the right auricular clot, and the numerous tuberculous thrombi and hematogenous tubercles in the pulmonary vessels. Her estimate of the number of bacilli contained in the circulating blood is based solely on the number left in the venous blood after its return to the

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<sup>1</sup> Jour. Infect. Dis., 1916, 19, p. 260.

heart. The small number of bacilli found there is more an index of the number dropping out of the blood stream than of the number entering it.

A better estimate of the degree of bacillema in tuberculosis would be the examination of arterial blood and clots. Usually, however, the left ventricle and aorta are empty at autopsy, and the left auricle contains but a small amount of clot or blood. The problem can be approached, however, by the study of the bacillary content of the right heart in cases of miliary tuberculosis in which the primary focus is on the venous side of the circulation, as for instance in the cervical or mesenteric lymph nodes. We have recently had in this laboratory such a case, with primary tuberculosis of the cervical lymph nodes and an even more widely disseminated miliary tuberculosis (skin, endocardium) than in Wilson's case, with lesions in the same early stage. The case was one of chronic lymphatic-myelogenous leukemia with secondary tuberculosis.

#### THE CASE

A stationary engineer, aged 50 years, entered the clinic with a history of progressive enlargement of cervical lymph glands. On Sept. 10, 1915, an enlarged cervical gland was removed and sent to this laboratory for pathologic examination. Sections showed a lymphoid (aleukemic) hyperplasia strewn with young epithelioid noncaseating tubercles. During his stay in the hospital, up to his death on Oct. 11, 1915, a rapidly increasing number of white cells in the blood was observed; so that the final clinical diagnosis was given as acute miliary tuberculosis with acute leukemia.

The pathological diagnosis follows: Chronic lymphatic (mixed lymphatic-myelogenous) leukemia; primary tuberculosis of cervical glands; acute miliary tuberculosis of all organs, including skin; endocardial tubercles; multiple lymphomata of kidneys and liver; leukemic infiltrations of gallbladder and intestine; Roentgen-ray pigmentation of skin and atrophy of testes; chronic parenchymatous nephritis; chronic catarrhal gastritis; bronchopneumonia.

The marked lymphoid hyperplasia of lymph glands, particularly the cervical, the thickening of the capsule, the hyperplasia of the stroma and the periglandular infiltrations, the hyperplasia of the spleen and the bone marrow, the presence of lymphomata and leukemic infiltrations throughout the body—all indicate a chronic leukemic process. The early stage of the tuberculous lesions shows a later secondary infection beginning in the cervical glands and thence spreading by both hematogenous and lymphogenous metastases throughout the body. The small number of leukocytes in the circulating blood when the patient was first examined, could be explained as the result of a decrease caused by acute tuberculous infection (aleukemic stage). The increase in the number of leukocytes observed during the patient's stay in the hospital may be interpreted as a change from the aleukemic to the former leukemic condition, induced by the acute infection.

The right auricle and ventricle were dilated and partly filled with soft leukemic clots (chicken-fat clots), 90 c.c. in amount in all. Sixty cubic centimeters of the clot were fixed in alcohol and 30 c.c. in formol. A portion of

the alcohol-fixed clot, measuring 9 by 6 by 0.45 mm. (24.3 c.mm.), was imbedded in paraffin, and cut into 60 sections, 7.5 microns each in thickness. These sections were floated onto warm carbol-fuchsin, stained, decolorized, and washed without removing the paraffin, then floated onto slides, dried, the paraffin removed by warming and applying xylol, and the sections finally mounted in balsam.

The 60 stained sections were thoroughly studied in all parts on a movable stage. Only absolutely typical and unquestioned tubercle bacilli were counted. The fact that the clots were leukemic, made up almost entirely of white cells, with but few red cells and these hemolyzed by the alcohol fixation, made the search for tubercle bacilli much easier than was the case in formol-fixed clots. By means of the movable stage one slide could be thoroughly examined in 1 hour; 60 full hours were given to the examination of the 60 slides. Four absolutely typical tubercle bacilli were found which by size, morphology, and staining were identified beyond question; also 15 suspicious fragments, which were disregarded. The sections were well stained, contained little dirt, and few acid-resisting crystalline structures were to be seen. The 4 bacilli were in the focal plane of the section between the white cells, and could not have been technical artefacts or acid-resisting bacilli from water or air. They must have existed in the blood clot at the time of its formation. The portion of clot examined represented 0.027% of the entire clot obtained from the heart. An estimate of the number of bacilli occurring in the entire clot, should this ratio of occurrence persist throughout, would give 14,815 bacilli for the entire clot. Such a calculation, however, does not sufficiently consider the laws of chance, and must not be too strongly heeded. The fact remains that in a case of acute miliary tuberculosis 4 tubercle bacilli to 60 slides, or 1 to 15, were found in the venous blood of the right heart. This proportion, which is not uncommon in sputum examinations in incipient cases of pulmonary tuberculosis, or in examinations of urinary sediments, is not considered insignificant as a clinical diagnostic effort in such conditions.

Tubercle bacillemia is a common, if not universal, occurrence in cases of tuberculosis. Can it be demonstrated in blood drawn from the living patient? Probably only by examination of many slides, a laborious undertaking, perhaps more laborious and time-consuming than the examination of sputum and urinary smears in cases of suspected incipient pulmonary and renal tuberculosis, but nevertheless worth while in many cases. The examination of blood taken from the living patient will probably give results more frequently in cases in

which the primary focus is in the lungs or bronchial nodes; while in the cadaver the heart blood of the right side will contain more bacilli in those cases in which the primary lesion is giving off bacilli to the venous circulation. The report of Brandes<sup>2</sup> on the examination of blood from 40 cases of surgical tuberculosis with positive results in 45% of these cases (5 c.c. of blood treated by the Schnitter-Uhlenhuth antiformin method) strengthens the belief that the attempt may not be as difficult as it now seems. A suggested improvement consists in imbedding in paraffin the sediment obtained by the antiformin method (decantation method of paraffin imbedding, as for blood), and the examination of the stained sections rather than smears. Thus some of the technical artefacts would be avoided and labor saved.

<sup>2</sup> Deutsch. med. Wchnschr., 1913, 39, p. 1137.

## A MULTIPLE PIPET FOR THE COMPLEMENT-FIXATION TEST \*

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Those familiar with the technic involved in the application of the complement-fixation test in the diagnosis of the various infectious diseases, are well aware that much time is consumed in the process of measuring and distributing the necessary substances, as normal sodium-chlorid solution, complement, sensitized rabbit serum, and suspension of blood corpuscles.

The type of pipet generally utilized in the process consists of glass tubes of uniform diameter, graduated into tenths or hundredths of a cubic centimeter, and with a capacity of 1, 5, 10, 20, or 25 cubic centimeters. Pipets of this type are usually filled by suction with the mouth controlled with the index finger. Constant practice enables one to be very accurate in the measurement of fluids and to acquire a moderate degree of speed in performing the work. When only a limited use of the complement-fixation test is necessary, pipets of this character are very satisfactorily employed. When on the other hand many samples of blood serum are to be diagnosed, a different method of distributing the fluids is necessary, otherwise numerous operators are required.

The need of a more rapid manner of accurately performing this work was recognized by the pathological division of the Bureau of Animal Industry, after the adoption of the complement-fixation test for diagnosing dourine, since it not infrequently happens that as many as 1,000 samples of blood serum are tested daily.

After experimenting with various devices in an endeavor to simplify the process, a multiple pipet was designed as shown in the accompanying illustrations. It not only permits of indefinitely greater progress, with greater accuracy, but at the same time eliminates the severe eye strain resulting from constant reading of the more generally used type of pipet.

This apparatus (Fig. 1) consists of a horizontal glass tube 12 in. in length which communicates below with 12 vertical pieces of tubing equally spaced and about 4 in. in length. These tubes, which are of uniform caliber, are slightly contracted at their free ends. A single

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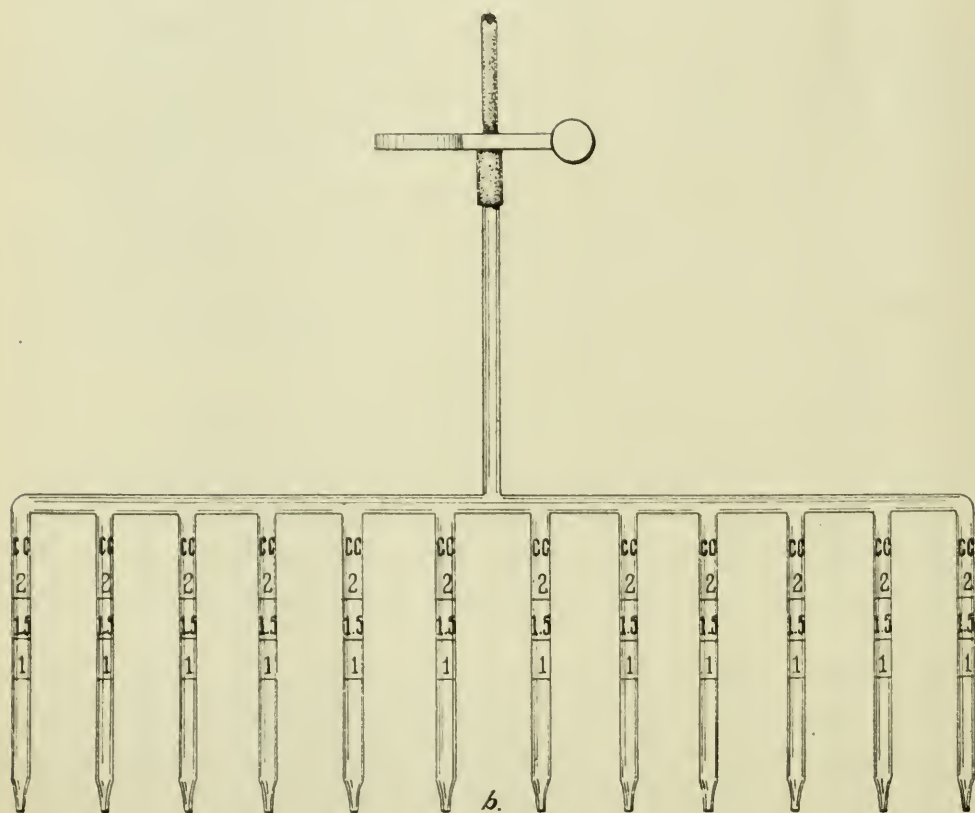
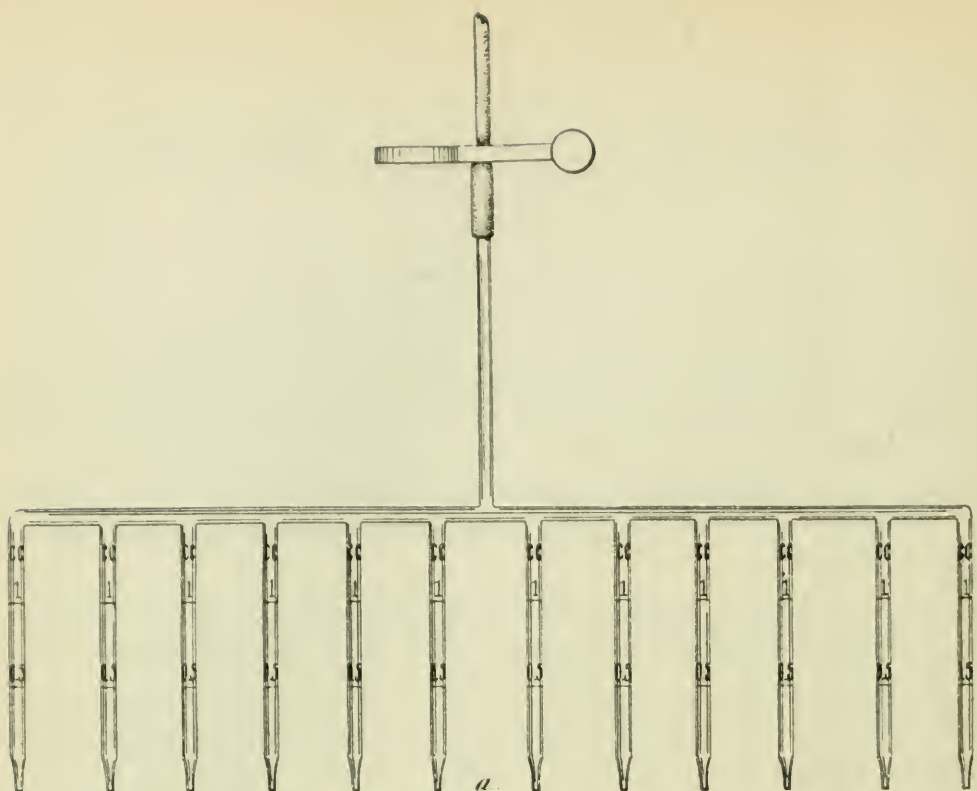


Fig. 1. Multiple pipet. (a) Utilized for distributing 0.5 c.c. or 1 c.c. (b) Utilized for distributing 1.5 c.c. or 2 c.c.

*James H. 1915.*

vertical section of tubing communicates with the horizontal part above and at the center.

The caliber of each of the vertical tubes dropped from the horizontal is such that when the pipet is immersed in a fluid to a depth of

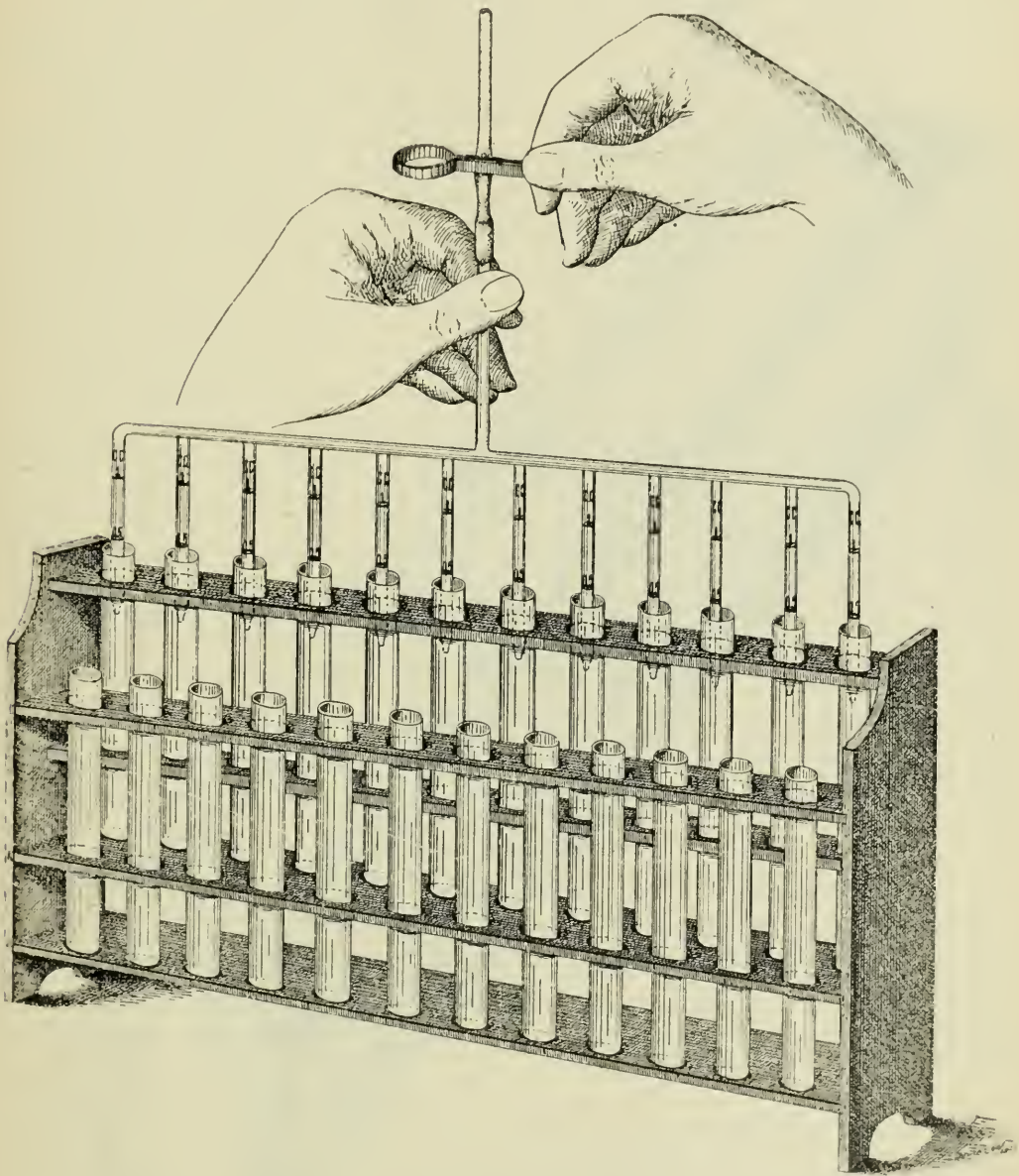


Fig. 2. The type of pipet used and the method of its operation.

about  $2\frac{1}{2}$  in., each tube contains 2 c.c. or 1 c.c., depending on which of 2 sizes of pipets is employed. The vertical tubes of the larger pipets are graduated at the points when the content is 2 c.c., 1.5 c.c., and 1 c.c., respectively; but when it is desired to distribute 1 c.c. or 0.5 c.c. of a

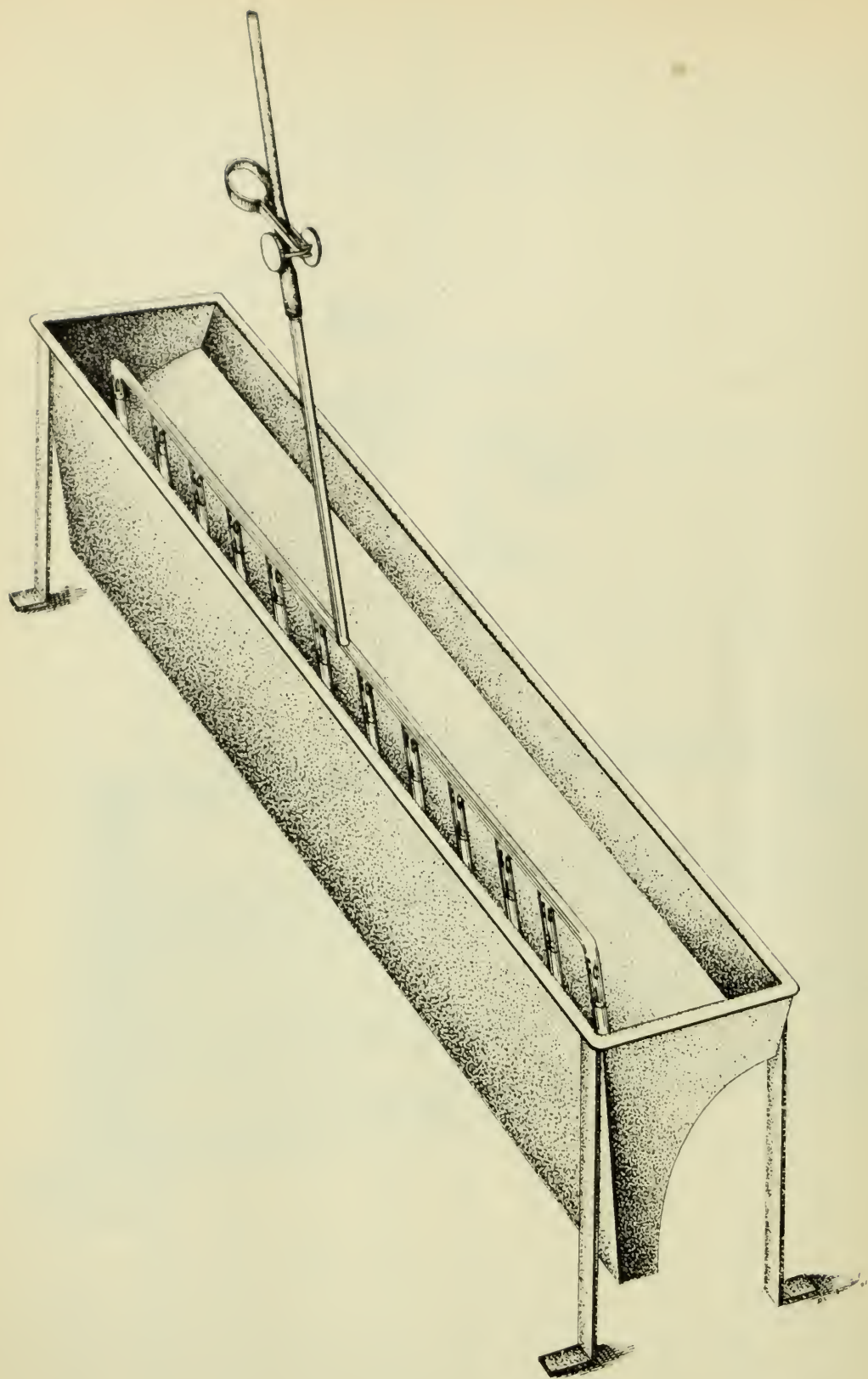


Fig. 3. Container for the substance to be distributed.

fluid, greater accuracy of measurement can be obtained through the use of the smaller caliber pipet, the diameter of the vertical tubes of which are such that when immersed in a fluid to a depth of about 2 in. the content of each is 1 c.c.

The pipet is controlled by means of a short piece of rubber tubing attached to the single vertical portion above the horizontal, to which a clamp is applied.

When operating, the clamp is released, the pipet immersed in the fluid to the 2-c.c., 1.5-c.c., or 1-c.c. graduation on the vertical tubes, and the clamp is then permitted to exert pressure on the rubber tube. The content of each vertical tube when the pipet is then removed from the fluid is 2 c.c., 1.5 c.c., or 1 c.c., depending on whether the rubber tube was clamped when the pipet was immersed to the 2-c.c., 1.5-c.c., or 1-c.c. graduation.

When pipets of this type are utilized, it is apparent that the test-tube racks should be constructed in such a manner that the spacing of the test tubes corresponds to the spacing of the vertical sections of the pipet; and it is advisable that the supporting bar for the upper portions of the tubes be placed not farther than 1 in. from the open end; otherwise some difficulty may be experienced in introducing the 12 vertical tubes of the pipet into the 12 test tubes for the delivery of the fluid. Figure 2 illustrates the design of rack employed. The 24 test tubes are so securely held that it is not necessary to remove and shake them separately when the various substances are added, but the rack may be shaken as a whole without danger of any of the test tubes being dislodged.

The receptacle used as container for the fluid that is to be distributed is illustrated in Figure 3. It is constructed with a narrow lower portion in order that suction with the mouth need be resorted to only in the distribution of the last 150 c.c. of the fluid, when its depth is not sufficient to fill the vertical sections of the pipet to the necessary level.

No great amount of practice is necessary in the manipulation of the apparatus to accomplish the ready and accurate distribution of 1 or 2 c.c. of a fluid to 1,000 test tubes in from 10 to 15 minutes.

Pipets of the content and graduation described are designed for use when the technic employed in applying the complement-fixation test is similar to the original Wassermann method, where the total quantity of the substances employed in combination with normal sodium-chlorid solution in each test tube is 5 c.c.



# COMPARISON OF A RAPID METHOD OF COUNTING BACTERIA IN MILK WITH THE STANDARD PLATE METHOD\*

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Having described a rapid method of counting bacteria in milk by means of "lilliputian" plate cultures,<sup>1</sup> I propose here to discuss the results obtained by this method in the examination of a series of milks during the past year. The methods employed will again be described, since they differ essentially from those previously used in milk analyses.

## THE RAPID METHOD OF COUNTING BACTERIA IN MILK

In the usual, or standard, method of milk analysis, a very small fraction of a cubic centimeter of milk is plated, and incubated long enough for the bacteria to grow into colonies visible to the naked eye. In my method a comparatively low dilution of milk is made with nutrient agar, spread over a definite area on a microscopic glass slide, and incubated only until the small colonies are visible under a compound microscope. These little colonies are then rendered prominent and easy to count by the following method of staining. The culture is dried down, fixed in the flame, treated with 10% acetic acid in alcohol to prevent the agar from firmly binding the stain, and stained with a 1:4 dilution of Loeffler's methylene blue, applied for 2 minutes. The stain is then partially washed out in alcohol or water and the preparation dried for examination. The colonies are a deep blue, while the agar background is only tinged or quite clear. (Fig. 1).

The counting is done under the low power of a compound microscope, a 2/3 or 1/2 inch (16 or 12 mm.) objective and an eyepiece of medium power.

To determine the area of the microscopic field the diameter is measured with a stage micrometer and the formula  $\pi R^2$  applied. The area of the little plate is 400 sq. mm. (2 by 2 cm.), and the area of the microscopic field 2 mm. with a common combination of lenses. Thus the area of the plate becomes 200 times that of the microscopic field. The number of colonies in a field of the microscope, multiplied by 200, and again multiplied by the dilution of the milk used in making the plates, gives the number of bacteria per cubic centimeter of milk. At least 20 fields should be counted and these should be selected mechanically to avoid errors proceeding from the personal equation.

## ANALYTIC RESULTS

Comparative analyses were made on 37 milks. Most of the samples came from the university creamery or the university barn. At the

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<sup>1</sup> Science, 1915, 42, p. 255. Jour. Am. Med. Assn., 1916, 66, p. 889.

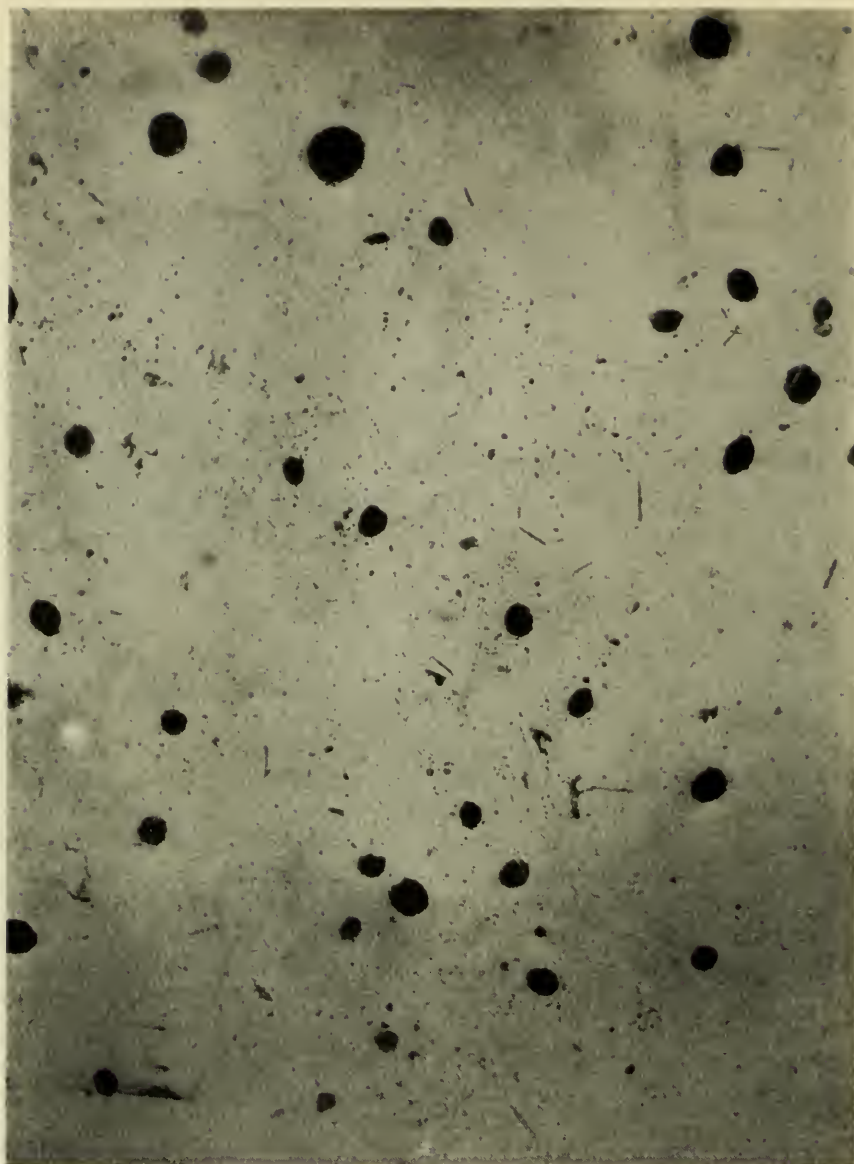


Fig. 1. A microscopic field showing colonies on the little plates after incubation period of  $4\frac{1}{2}$  hours.  $\times 30$ .

creamery the milk was obtained directly from the farmers, some of it having been brought a considerable distance. In some cases it was examined immediately upon arrival, in which case it represented ordinary market milk, and in other cases it stood about the laboratory some hours before analysis, with the result that its bacterial count was high. The milk from the university barn, as it was produced under good conditions, usually had a low bacterial content. Single or at most a few samples of other milks were used from time to time as occasion offered.



Fig. 2. Microscopic field showing a few of the same colonies seen in Fig. 1.  $\times 225$ .

Parallel analyses were usually made by the same person, but in some cases the standard plates were made by others and the little plates by ourselves.

*Methods used.*—The plate cultures were made in the usual way according to the standard methods. The milk was first thoroughly shaken, and then 1 c.c. was removed for analysis. The water blanks, made from tap water sterilized in the autoclave, contained either 9 or 99 c.c. The agar in my own work was made according to the standard methods and had a reaction of +1. The period of incubation was 48 hours at 37 C. In most of the samples the counting was done in 3 ways; first, with the naked eye, again, under a reading glass,

and finally, with the lens suggested by the committee on milk, of the American Public Health Association.<sup>2</sup> Whenever possible all the colonies on the plate were counted. Several dilutions were always made and usually all dilutions were counted, and the results averaged.

The little plates were made by putting 1 c.c. of the milk, after thorough shaking, into the agar directly, or after dilution with sterile tap water. The dilutions most frequently employed were 1:20 and 1:200. Attention is called to the fact that these small dilutions are used even with highly contaminated milks.

The counts of the little plates were made under 3 different magnifications—with a 16-mm. lens, a 6-mm. (1/4 in.) lens, and a 2-mm. lens. Several plates were usually counted for each sample.

A marked difference existed between the counts obtained under different magnifications. With the lowest power some of the minute colonies were missed, while with the higher dry power all the colonies were seen, as well as groups of dead bacteria which were not readily distinguished from colonies of growing bacteria. With the oil-immersion objective, all the groups of bacteria were counted, these counts naturally being higher than those secured by either of the other methods.

*Milks Studied.*—The milks examined were classified according to the number of bacteria contained, as follows: Class A, milks containing less than 10,000 bacteria per cubic centimeter (Samples 32, 35, 37, 23, 38, 25, and 24). Class B, milks with a count of between 10,000 and 100,000 bacteria per cubic centimeter (Samples 4, 3, 15, 29, 30, 6, 36, 10, 33, 31, 19, 39, and 12). Class C, milks with a count of between 100,000 and 1,000,000 bacteria per cubic centimeter (Samples 5, 11, 8, 28, 15, 27, 21, 26, 16, and 22). Class D, milks containing over 1,000,000 bacteria per cubic centimeter (Samples 1, 2, 18, 14, 20, and 17).

*Bases of Comparison.*—With both methods 3 counts were made. With the standard plates, these counts came from the different degrees of magnification used in counting the colonies. In comparing the two methods the count obtained by means of the highest magnification, when it was available in the standard plates, was compared with the count secured through the use of the low-power lens (16 mm.). These figures gave the most comparable results.

The milks belonging to Class A, with a bacterial count of from 0 to 10,000, gave the results recorded in Table 1.

Table 1 shows that counts obtained by the two methods parallel each other closely. The counts with both methods fell into the same class, and the order was the same in both cases, with the exception of Nos. 4, 5, and 6. The numbers obtained with the little plates were always larger, being on an average 60% higher. The ratio between the two counts varied from 1.10 to 2.86, as compared with 1. The percentage of variation between the results with the little plates of the same count was without exception less than the variation between the counts obtained with standard plates. This indicates that the results obtained by the rapid method are quite as reliable as those obtained by the standard-plate method, even tho somewhat higher.

<sup>2</sup> Am. Jour. Pub. Health, 1915, 5, p. 64.

TABLE 1  
CLASS A, WITH COUNTS OF LESS THAN 10,000

	Serial Number	Standard Plates			Small Plates			Ratio Between Counts
		Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	
1	34	4	675	25	4	945	14	1:1.25
2	32	6	940	48	6	1,080	20	1:1.14
3	35	6	2,625	60	6	2,925	8	1:1.11
4	37	5	2,900	64	6	5,165	16	1:1.78
5	23	2	3,000*	10	2	4,900	2	1:1.63
6	38	6	2,950	78	8	8,440	26	1:2.86
7	25	4	4,900*	19	4	9,500	5	1:1.94
8	24	2	8,300*	10	3	9,200	6	1:1.10
Average.....			3,286	39	...	5,267	12	1:1.60

\* Plates counted with the naked eye only.

A comparative study of the milks with a bacterial count of from 10,000 to 100,000 on the standard plates gave the results shown in Table 2.

TABLE 2  
CLASS B, WITH COUNTS BETWEEN 10,000 AND 100,000

	Serial Number	Standard Plates			Small Plates			Ratio Between Counts
		Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	
1	4	2	15,500	3	2	8,200†	2	1:0.52
2	3	2	16,000	6	2	31,350	17	1:1.90
3	15	4	23,750	16	4	23,000	11	1:1.00
4	29	6	31,000*	35	2	99,000	5	1:3.12
5	30	4	34,000	36	2	75,000	0	1:2.20
6	6	3	34,000	50	2	20,000	.0	1:0.58
7	36	2	40,000	25	3	13,400	11	1:0.32
8	10	4	41,000	23	4	22,000	12	1:0.53
9	33	4	42,500	30	4	42,400	11	1:1.00
10	31	6	46,000	25	4	50,000	12	1:1.08
11	19	4	51,000	35	3	14,800	17	1:0.27
12	39	6	75,000	37	3	72,630	7	1:0.96
13	12	3	96,700	73	4	17,000	21	1:0.17
Average.....			42,030	30	...	37,537	9.7	1:0.89

\* Counted with naked eye only.

† Counted under the oil-immersion objective.

Table 2 records that the fluctuations were greater in this group than in the former. The ratio between the two counts varied from 0.17 to 3.12 as compared with 1. On the whole the number of bacteria found on the little plates was slightly less than the number found on the standard plates. The percentage of variation from the average count for the standard plates was higher than the percentage of variation

from the average for the little plates; that is, as 9.7 is to 30, or more than threefold.

The results for the milks the bacterial counts of which varied between 100,000 and 1,000,000 per cubic centimeter on standard plates are recorded in Table 3.

TABLE 3  
CLASS C, WITH COUNTS BETWEEN 100,000 AND 1,000,000

	Serial Number	Standard Plates			Small Plates			Ratio Between Counts
		Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	
1	5	4	110,000	45	1	28,300	0	1:0.25
2	11	4	129,500	65	4	35,500	45	1:0.27
3	8	4	148,000	18	3	206,000	9	1:1.32
4	28	5	166,000*	30	3	650,000†	17	1:3.31
5	13	3	171,000	23	2	225,000	12	1:1.31
6	27	5	293,000*	77	2	119,000	6	1:0.40
7	21	6	329,700*	18	4	610,000†	18	1:1.92
8	26	4	350,000*	27	2	103,000	3	1:0.29
9	16	4	474,000	10	4	134,000	2	1:0.29
10	22	3	503,000*	29	4	3,742,000†	16	1:4.14
Average.....			307,000	34	...	585,200	13	1:1.90

\* Standard plates counted with naked eye only.

† Little plates counted with high dry power only.

In Table 3 it is seen that the comparative results were reasonably close. The ratio between the two counts varied from 0.25 (No. 1) to 4:14 (No. 10) as compared with 1. . On the whole, the number of bacteria found by means of the little plates was higher than that found on the standard plates. The ratio was 1.9 for the little plates and 1 for the standard plates. The samples showing the greatest variation were those in which the data were incomplete; for example, Nos. 4, 7, 8, 9, and 10. It will be shown that the count on the standard plates is lower with the naked eye than with the lens, and that the count on the little plates is higher with a  $\frac{1}{4}$  inch lens than with a  $\frac{2}{3}$  inch lens, so that in these samples the data used tend to separate the samples, and may account for the high degree of variation. The percentage of variation from the average count was 34 in the case of the standard plates, and 13 for the little plates, indicating that the variation in the results from the different plates for each method is only one-third as great in the case of little plates as in the case of the standard plates.

The results obtained with milks varying in bacterial count from 1,000,000 to 10,000,000 per cubic centimeter (Class D) are given in Table 4.

TABLE 4

CLASS D, WITH COUNTS BETWEEN 1,000,000 AND 10,000,000

	Serial Number	Standard Plates			Small Plates			Ratio Between Counts
		Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	
1	1	2	1,250,000*	12	2	1,160,000†	3	1:0.93
2	2	2	1,630,000	5	2	1,400,000	14	1:0.86
3	18	4	2,500,000	19	4	1,254,000‡	23	1:0.50
4	14	2	4,000,000	25	1	2,232,000	0	1:0.55
5	20	4	7,760,000	16	4	1,614,000‡	9	1:0.20
6	17	4	20,750,000	37	2	15,930,000‡	2	1:0.72
Average.....			6,315,000	19	...	3,931,700	10	1:0.62

\* Counted with naked eye only.

† Counted under oil-immersion objective.

‡ Counted under high dry power only.

The counts in this group demonstrate that the little plates give uniformly lower counts than the standard plates. The ratios between the two counts, No. 5 excepted, were very close, the average ratio being 1:0.62. The percentage of variation from the average was twice as great with the standard plates as with the little plates, that is, 19 to 10.

## RESULTS AS A WHOLE

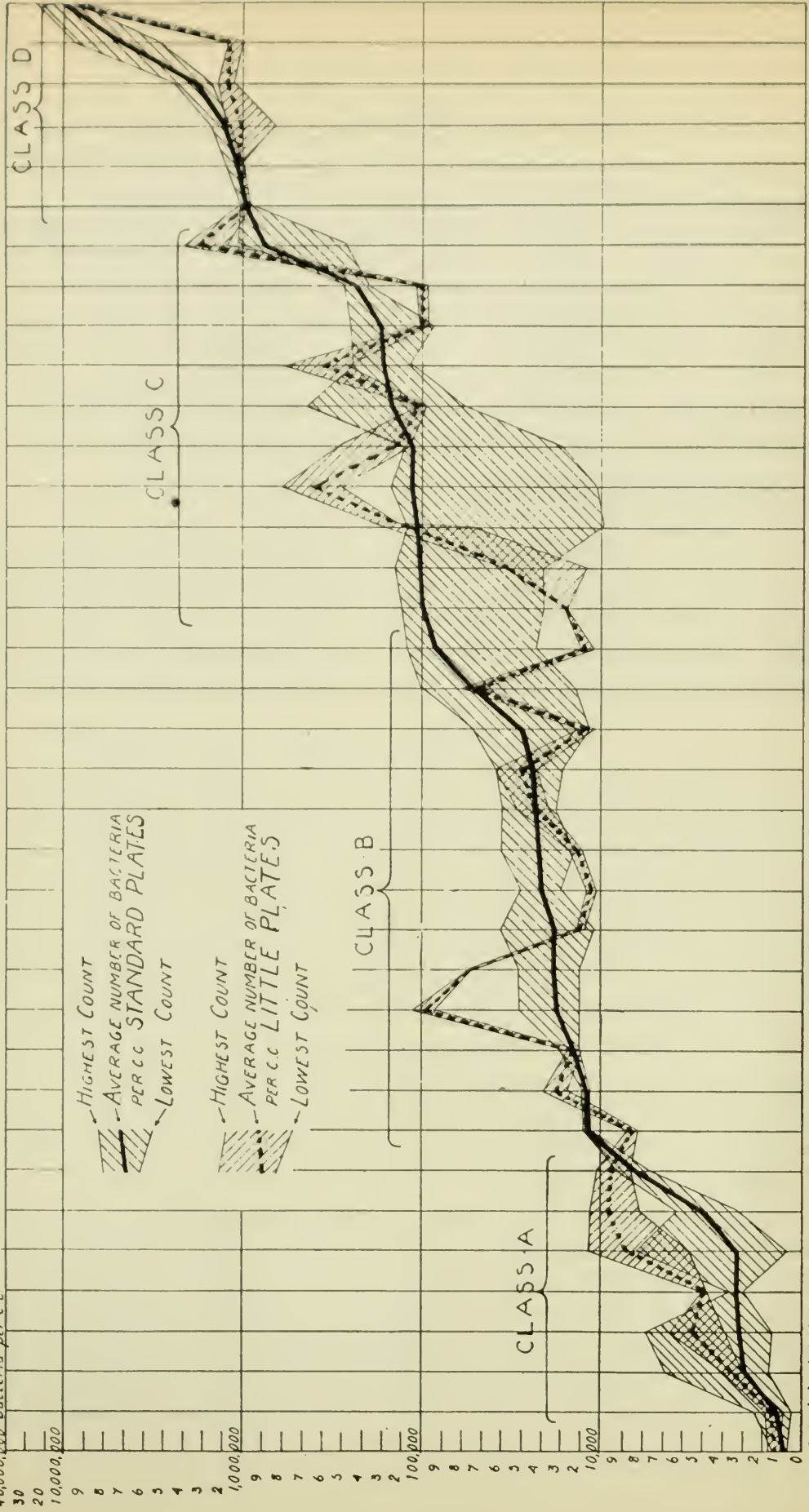
The results of the analyses recorded here are shown in the accompanying chart. The bacterial count, as determined by the standard-plate method is represented by the heavy continuous line. The maximal variations above and below the average are indicated by cross-hatching, accompanying the line, and extending up and to the right. The count of the same milks as determined by means of the little plates is shown by the broken line. The maximal variation from this average is represented by cross-hatching drawn down and to the right.

The scale at the left indicates the bacteria per cubic centimeter. Equal divisions on this scale represent an increase of tenfold at each horizontal line; i. e., in the lower tier the divisions equal 1,000, and in the upper tier 1,000,000. The numbers of the samples are indicated at the bottom of the chart.

On the whole, the correspondence seems reasonably close, and warrants the statement made in a previous communication, namely: "The results obtained indicate that the difference between the counts secured by the rapid method and the ordinary or standard method usually amounts to little more than the variation which occurs between duplicate plates, or between different dilutions in the same analysis by the ordinary plate method."<sup>3</sup> Since only the comparative variations are

<sup>3</sup> Frost: Science, 1915, 42, p. 255.

4,000,000 bacteria per c.c.



1(34) 2(32) 3(33) 4(37) 5(23) 6(38) 7(25) 8(24) 9(4) 10(3) 11(15) 12(24) 13(5d) 14(6) 15(36) 16(10) 17(53) 18(33) 19(19) 20(24) 21(12) 22(15) 23(17) 24(8) 25(18) 26(18) 27(6) 28(12) 29(14) 30(14) 31(22) 32(12) 33(12) 34(12) 35(14) 36(14) 37(7)

correctly visualized in the chart, the variations appear to be greater in Class A than in Class D, tho in reality they were smaller. The actual variations are recorded in the tables.

# COUNTS AT DIFFERENT MAGNIFICATIONS

## A. STANDARD PLATES

The number of bacteria obtained per cubic centimeter of milk varies with the magnification used in counting the colonies. This is in particular true of the little plates, tho it also applies to the standard plates.

Various counting devices are recommended by different workers. In Standard Methods for the Bacterial Examination of Milk<sup>4</sup> it is recommended that "colonies too small to be seen with the naked eye or with slight magnification shall not be considered in the count." There are several counting apparatuses which use a lens of low power; e. g., a reading glass about 10 cm. in diameter. The committee on milk of the laboratory section of the American Public Health Association<sup>2</sup> proposes that the counting be done with a lens of 3½ diameters magnification and recommends an engraver's lens (B. & L. 146).

Table 5 records the results which I have obtained using the different methods of counting.

TABLE 5  
VARIATION DUE TO THE METHOD OF COUNTING STANDARD PLATES

Serial No.	Naked Eye	Reading Glass	× 3½	Average Ratio Between Counts
Class A				
34	650	650	675	
32	625	880	940	
35	2,400	2,475	2,600	
37	2,500	2,800	2,900	
38	2,166	2,700	2,950	
25	4,800	4,800	4,900	
Average	2,190	2,384	2,494	1:1.09 : 1.14
Class B				
15	17,750	18,500	23,750	
30	16,250	18,750	34,000	
36	35,000	35,000	40,000	
33	37,500	41,500	42,500	
31	45,000	46,000	46,000	
19	42,000	43,000	51,000	
39	72,500	75,300	75,300	
12	58,000	91,700	96,700	
Average	40,500	46,220	51,156	1:1.14 : 1.26
Class C				
11	112,500	122,500	129,500	
13	142,000	161,000	170,000	
16	283,000	281,500	474,000	
Average	179,166	188,333	257,833	1:1.05 : 1.44
Class D				
18	1,870,000	2,125,000	2,500,000	
14	6,000,000	3,880,000	4,000,000	
20	6,740,000	7,150,000	7,760,000	
17	17,000,000	18,325,000	20,750,000	
Average	10,536,666	10,493,377	11,670,000	1:0.99 : 1.10

\* Am. Jour. Pub. Hygiene, 1910, 6, p. 15.

In all cases save one (No. 14, Class D) the counts secured by the use of a lens magnifying  $3\frac{1}{2}$  diameters were higher than those obtained with a lower magnification or with the naked eye. The variation is not great, however. If the average for naked-eye counts is taken as 1, the average for those made under the reading glass becomes 1.07 and for those made with the engraver's lens 1.23. The results obtained bear out the contention of the committee<sup>2</sup> on milk that if the true or maximal count is sought, counts made with a lens magnifying  $3\frac{1}{2}$  diameters are more accurate than those made at lower magnifications. These higher counts have been selected, when possible, to compare with those obtained by the little plate method.

TABLE 6

COMPARISON OF COUNTS OBTAINED WITH THE RAPID METHOD AT VARIOUS MAGNIFICATIONS

Milk	Eyepiece 3 With			Average Ratio Between Counts
	Objective $\frac{2}{3}$ in. (82 $\times$ )	Objective $\frac{1}{4}$ in. (200 $\times$ )	Objective $\frac{1}{12}$ in. (840 $\times$ )	
Class B	23,000	43,000	70,000	1 :2.25:6.13
	99,000	200,000	290,000	
	75,000	54,500	104,000	
	20,000	112,000	200,000	
	22,000	44,000	125,000	
	42,400	128,500	745,000	
	50,000	96,000	125,000	
	14,800	58,700	87,000	
	72,630	188,000	793,000	
	17,000	56,000	132,500	
Average	43,580	98,070	267,150	
Class C	35,300	55,500	200,000	1 :1.93:3.21 1.00:2.09:4.67
	206,000	260,000	430,000	
	225,000	234,000	480,000	
	119,000	490,000	600,000	
	103,000	400,000	600,000	
	134,000	152,000	335,000	
Average	137,050	265,250	440,833	

## B. LITTLE PLATES

The proper magnification in counting the little plates is a matter of importance because of the marked increase in count which followed the use of the higher powers, as demonstrated in Table 6. If the count obtained by the low-power 16-mm. lens be taken as 1, the 6-mm. lens ( $\frac{1}{4}$  inch) gives a little over twice as many colonies, (2.09) and the oil immersion objective gives over  $4\frac{1}{2}$  times as many (4.67). Two reasons for this disparity appear: The small colonies may be missed under the low power, and readily seen under the higher powers; and the high powers, especially the oil-immersion objective may reveal

groups of dead bacteria which cannot readily be distinguished from colonies. The low-power lens gives a count that, while too small, is the most comparable with that from the standard plates. The error due to the multiplication factor becomes smaller, offsetting the advantage of finer distinctions offered with the higher powers. A lens giving a magnification of 200 instead of 84 would seem the most desirable, and such a lens is now being tried out. The length of the incubation period of the colonies is important, because the older the colonies are, the larger they become and the less likelihood there is of confusing them with clumps of dead bacteria. Further work seems to show that when the period of incubation of the colonies in the little plates is from 6 to 8 hours, comparable counts are obtained with the different magnifications.

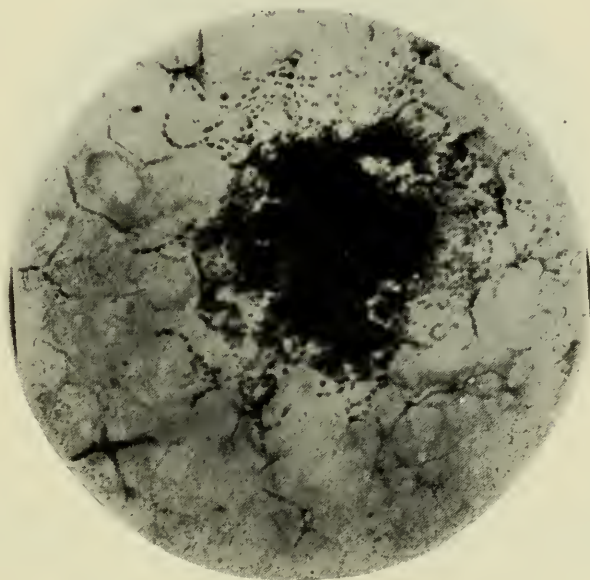


Fig. 3. A colony .5 hours old under the oil-immersion objective showing the individual cells and the arrangement during growth—probably *Bacillus acidilactici*. The reticulated structure of the medium is due to the fat globules in the milk which was used with the agar, which also accounts for the open places in the colony.  $\times 750$ .

#### PERIOD OF INCUBATION

The chief merit of, and the principal purpose in mind in developing this method of analysis was that it requires but a short period of incubation. Forty-eight hours are needed for the standard method. The period of incubation for the little plates may be reduced to 3 hours in some cases, 6 hours in most cases, and from 8 to 12 hours in all cases. Unless there is imperative need of reaching the results in less time, it is desirable to incubate not less than 6 hours in all cases. When the milk is fresh from the cow, or has just been pasteurized, it contains

very few bacteria, necessitating an incubation period of 12 hours, as the bacteria grow into visible colonies very slowly. With this limitation, the method can be used for the examination of certified and other milks with a low bacterial count. Market milks, or those having a high bacterial content, produce colonies in a remarkably short time, a 3-hour period of incubation being sufficient to obtain a large part of the colonies. A few extra hours for the incubation of the plates can be taken however even in such milks as these, indeed, they can also be left for 18 or more hours without any detriment except the formation of spreaders, which do not interfere with the use of the plates.

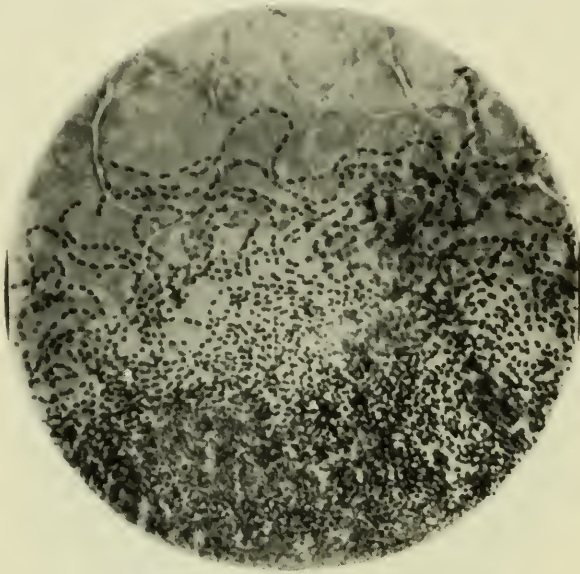


Fig. 4. The edge of a colony, showing individual bacteria and their arrangement in chains. This is probably a streptococcus colony.  $\times 750$ .

Most of the plates which were counted for the results recorded in this work were incubated for 5 hours or less, except those from the very good milk in Class A, which were incubated for 18 hours, altho 12 hours or less would have brought practically the same results.

#### ERRORS INHERENT IN THE METHODS OF ESTIMATING THE NUMBER OF BACTERIA IN MILK

In the standard method the chief sources of error lie in the dilution of the milk and the methods of counting. In the process of dilution many factors are variables,<sup>5</sup> but even granting that all the factors in the process are constants, the high dilution to which milk must frequently be subjected, magnifies inherent difficulties in sampling, mea-

<sup>5</sup> Frost: Tr. Wisconsin Acad. Sciences, 1914, 17, p. 1306.

suring, etc.; hence it is plain that bacterial counts of the same milk vary when the results are found by multiplying a small number of colonies actually counted by a high dilution factor. In addition the errors which must occur when a portion only of the colonies growing on a plate are counted, and counted under various degrees of magnification, further the divergencies in parallel determinations.

Some of these variable factors are avoided with the little plates. The dilution, for example, need not be over 1:200 as compared with the dilutions of 1:100,000, or 1,000,000 which are sometimes used. In the rapid method a new factor, already referred to as the microscope



Fig. 5. The edge of a colony found in milk showing a long square-end bacillus which grew very rapidly.  $\times 750$ .

factor, is present, and that is the quotient obtained by dividing the area of the little plates by the area of the microscopic field. For the microscope and magnification used ( $\frac{2}{3}$  in. objective), this is 200; for the  $\frac{1}{4}$ -inch objective 2,000, and for the oil immersion 20,000.

#### CULTURE MEDIUM

The amount of milk added to the agar, particularly in the lower dilutions, is large and changes the nature of the culture medium. This might serve to influence the bacteria which develop, and cause parallel counts obtained by different methods to diverge widely. However, there might be sufficient nourishment in the milk added to permit the substitution of a simple agar solution for the nutrient agar, thus simplifying the technic by obviating the making of culture media. In this

case, all dilutions of the milk to be analyzed would have to be made in sterile milk instead of water. As yet, no definite data on this point have been gathered except that results have not been such as to warrant a departure from the original technic of using nutrient agar.

#### DIFFERENTIATING BACTERIA BY MEANS OF THEIR MICROSCOPIC COLONIES

With the higher powers of the microscope the small colonies may readily be observed and the different micro-organisms recognized through their peculiarities, as shown by the photomicrographs. From a single preparation, then, the number of bacteria present can be determined, and individual micro-organisms recognized.

#### ADVANTAGES OF THE LITTLE-PLATE METHOD

The method is rapid, requiring only from one-twelfth to one-fourth of the time needed with the standard-plate method.

Its technic is simpler, requiring less glassware and culture medium.

It furnishes a means of keeping a permanent record since the slides can be filed.

By proper methods of staining, the individual bacteria or groups of the same that have not grown, can be seen and thus the total number of bacteria in the milk determined. This is not possible by the standard-plate method.

Then there is the advantage of being able to see whether or not the bacteria are alive, making it possible to study the germ content of pasteurized milk and to distinguish dead bacteria from the living, as cannot be done with the direct microscopic examination such as that recommended by Breed.

#### SUMMARY

Thirty-seven samples of milk varying in bacterial content from 675 to 20,000,000 bacteria per cubic centimeter were compared. Each sample was analyzed by the standard-plate and by the little-plate method, and a satisfactory correspondence between the results was found. With the exception of 2 samples, all were placed in the same classes by both methods. If the result obtained by the standard plates is taken as 1, the result, for the milks in Class A for the little plates, was 1.6; for Class B, 0.89; for Class C, 1.9; and for Class D, 0.62.

The results varied considerably with the different magnifications used in counting the colonies. Greater variations existed with the little

plates than with the standard plates, because under the higher powers groups of bacteria, dead or not yet grown into colonies, were counted.

The necessary period of incubation for the little plates varies from 3 to 12 hours.

A much larger amount of milk is used with the little plates than with the standard plates, hence the former method ought theoretically to be the more accurate of the two. The different counts on the same milk in the little plates always showed less variation among themselves than did those on the separate standard plates.

The rapid method permits the ready examination of young colonies of bacteria, and to this extent offers an important means of identifying particular bacteria.

# ADAPTATION TO CERTAIN TENSIONS OF OXYGEN AS SHOWN BY GONOCOCCUS AND OTHER PARASITIC AND SAPROPHYTIC BACTERIA \*

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Practically all our knowledge concerning the respiration of bacteria has been thoroughly reviewed by Kruse,<sup>1</sup> and an especially illuminating discussion of this subject has been given by Meyer.<sup>2</sup> We shall not go into detail concerning the influence of oxygen tension on sporulation, germination, division, and motility. We would remind the reader, however, that chiefly through the investigation of Chudiakow, Winogradski, Porodko, Wundt, and others, it became known that the optimal conditions for the growth of any single species do not depend so much on the presence or absence of oxygen as on its tension, and further, that through the work of Beijerinck<sup>3</sup> we became acquainted with a group of bacteria which thrive only at a partial atmospheric tension of oxygen—the microaerophiles. Among these may be mentioned the bacteria studied in bean infusions by Beijerinck; the sulfur bacteria, the peculiar behavior of which was reported by Winogradski; *Amylobacter butylicum*, which, according to Beijerinck's observation, only grows and ferments wort under anaerobic conditions but grows better at a lower temperature in peptone-starch water under more nearly aerobic conditions; *B. abortus*, which von Bang and Stribolt found required a partial tension for its growth; a bacillus isolated from fowl diphtheria by Müller;<sup>4</sup> and certain pyogenic streptococci, as shown by Wittneben.<sup>5</sup>

It seems remarkable to us that greater attention has not been paid to the oxygen requirements of parasitic bacteria. A few recent experiences have suggested to us that the cultivation of many of the unknown viruses of infectious diseases may depend more on the presence of the right oxygen tension than on the composition of the

\* Received for publication May 26, 1916.

<sup>1</sup> Allgemeine Mikrobiologie, 1910.

<sup>2</sup> Centralbl. f. Bakteriöl., I, O., 1909, 49, p. 305.

<sup>3</sup> Ibid., 1893, 14, p. 827.

<sup>4</sup> Ibid., I, O., 1906, 41, pp. 521, 621.

<sup>5</sup> Ibid., 1907, 44, p. 96.

artificial medium. Rosenow,<sup>6</sup> however, has repeatedly noted that cultures made from rheumatic joints, appendicitis, etc., are more apt to yield positive results when tall columns of broth or agar are used. Of cultures from rheumatic joints he says: "That the oxygen requirement is the chief factor to explain this difference in my results and the negative results of others is indicated also by the fact that the colonies never developed above 0.5 cm. from the top and never below 2 cm. from the bottom of the agar tubes. The largest number of colonies developed between 1.5 cm. from the top and 3.5 cm. from the bottom." This oxygen-tension requirement was lost in the Rosenow strains on further cultivation (presumably aerobic or anaerobic subcultures) and he further notes<sup>7</sup> that "after cultivation from one to eight months, the capacity to grow at a low temperature, the sensitiveness to oxygen, the excessive production of acid in dextrose broth, and the simultaneous affinity for joints, endocardium, and myocardium are found to have largely or entirely disappeared." Again, in connection with bacteriologic examination of the glands draining the lesions in arthritis deformans Rosenow states that "all the streptococcal forms isolated have shown a marked preference for anaerobic conditions of growth in the primary culture."<sup>8</sup>

We record here the details of our discovery<sup>9</sup> that the gonococcus is a partial-tension organism. We also describe a partial-tension clostridium; and a partial-tension bacterium, from a human knee joint, resembling *B. abortus*. None of these three organisms will grow anaerobically, but they throw off aerobic variants from their partial-tension growths. We further show that *Leptothrix innominata* of the human mouth has a very wide range of oxygen tension and we record some observations which tend to show that *B. typhosus* becomes adapted to partial-tension growth within the body.

#### TECHNIC

We have produced partial oxygen pressure in our cultures almost entirely by the method first suggested by Nowak,<sup>10</sup> but instead of sealing the cultures in a jar along with one or more slant cultures of *B. subtilis*, we have attached a recently inoculated slant culture of this organism to the tube in which partial tension is required, by means of rubber tubing. Modifications of Nowak's method have been suggested by Horton<sup>11</sup> and Giltner.<sup>12</sup> It will also be recalled

<sup>6</sup> Jour. Infect. Dis., 1914, 14, p. 62.

<sup>7</sup> Ibid., p. 66.

<sup>8</sup> Jour. Am. Med. Assn., 1914, 62, p. 1146.

<sup>9</sup> Wherry and Oliver: Lancet-Clinic, 1916, 115, p. 306.

<sup>10</sup> Ann. de l'Inst. Pasteur, 1908, 22, p. 541.

<sup>11</sup> Jour. Infect. Dis., 1914, 15, p. 22.

<sup>12</sup> Centralbl. f. Bakteriöl., R., 1914, 63, p. 522.

that Theobald Smith and Fabyan<sup>13</sup> showed that other bacteria—*B. megatherium* and *B. coli* for example—could be used in place of *B. subtilis*. Our method is an easy one and enables one to re-attach freshly inoculated tubes of *B. subtilis* at various intervals. Micro-organisms which grow slowly may not appear at all unless *B. subtilis* tubes are changed daily so as to maintain the required tension. In this connection it is well to remember that *B. subtilis* may still multiply at an atmospheric pressure of 10 mm., tho not at 5 mm. (Chudiakow).

In making partial-tension plates we have inverted the inoculated plate upon a glass plate on which is fastened (with a small piece of plasticine) a smaller open Petri dish containing a freshly inoculated culture of *B. subtilis*. The inverted dish should be high enough to avoid any contact with the smaller dish. The edges of the inverted dish are then sealed with plasticine as recommended by Lentz<sup>14</sup> for the growth of anaerobes. Plates made in this way and sealed to pieces of window glass of suitable size, may be stacked in the incubator.

#### THE GONOCOCCUS A NATURAL MICROAEROPHILE

The recognition of the fact that the gonococcus is a partial-tension organism renders its cultivation an easy matter. In a study of vulvovaginitis we had the usual experience with a variety of media but were finally successful in obtaining aerobic strains of the gonococcus from 4 cases of vulvovaginitis in children and from the urethral pus of an adult male. Having been particularly impressed with the work of W. Blair M. Martin,<sup>15</sup> we used the medium and criteria established by him for the cultivation and recognition of the gonococcus. We were disappointed, however, in invariably obtaining only a few colonies of the gonococcus when the inoculated pus showed that myriads of organisms were present. The following experiences which we have had more recently, explain the discrepancy.

(a) Boy, 4 years old. Acute purulent urethritis with extreme phimosis, which had begun one week before. Smears showed numerous intracellular gram-negative diplococci resembling gonococci. One loopful of pus was smeared by the successive-stroke method over 3 slants of +0.5 Martin's medium containing sterile pleuritic fluid. Two sets of 3 tubes each were inoculated in this way. One tube which was inoculated with a loopful of pus, was kept at partial tension. Incubation 37 C.

In 24 hours the partial-tension tube showed hundreds of minute translucent gonococcus-like colonies, which when subcultured corresponded culturally to the gonococcus. None of the aerobic tubes showed any growth in 24 hours and no gonococcus-like colonies

<sup>13</sup> Centralbl. f. Bakteriöl., I, O., 1912, 61, p. 549.

<sup>14</sup> Ibid., 1910, 53, p. 358.

<sup>15</sup> Jour. Path. and Bacteriol., 1911, 15, p. 76.

appeared at all, tho in from 48 to 72 hours there were a few colonies of the urethral diphtheroid.

Two days later this experiment was repeated with in each case one loopful of the urethral pus for the inoculation of each slant. No gonococcus-like colonies appeared on the aerobic slants, but on slants kept at partial tension they were very numerous in 48 hours.



Figure 1

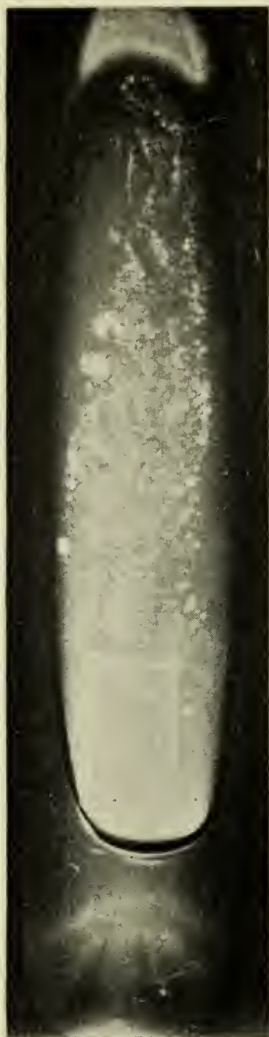


Figure 2



Figure 3

Figs. 1 and 2. Aerobic and partial-tension cultures from the male urethra in gonorrhea. One loopful of pus spread on each slant. Incubation on + 0.5 Martin's pleuritic medium for 24 hours at 37 C. The vast majority of the colonies on the partial-tension slant (2) are gonococcus colonies.  $\times \frac{1}{2}$ .

Fig. 3. Isolated gonococcus colonies on + 0.5 Martin's pleuritic medium, partial tension, at 37 C. for 4 days. Note delicate translucent edge. Natural size.

An aerobic strain of the gonococcus was derived from one of these partial-tension subcultures as follows:

Aerobic subcultures were made along with partial-tension subcultures 24 and 48 hours after isolation; the aerobic subcultures remained sterile during 8 days' observation. When the partial-tension strain was in its 8th subculture (8 days old), another attempt yielded an aerobic strain, which, however, grew rather poorly for the first two or three aerobic transfers and then grew as luxuriantly as the partial-tension strain. This aerobic strain was found to be dead after 6 days' incubation at 37 C.

We have not made many viability tests on the partial-tension strains, but one culture kept at partial tension for 15 days at 37 C., yielded almost as luxuriant a growth on subculture as one gets on transplanting a young culture; that is, an almost confluent layer. Another partial-tension strain isolated from a case of vulvovaginitis, after 19 consecutive days' incubation at partial tension at 37 C. yielded, on transplant, a luxuriant growth.

We have not succeeded in obtaining an anaerobic strain from the partial-tension cultures. Anaerobic subcultures kept for 4 days at 37 C. and then placed at partial tension do not yield growth.

The 7-year-old sister of this little boy, with whom he slept, had no vaginal discharge and aerobic and partial-tension cultures yielded no gonococcus-like colonies. The parents denied having, or having had, gonorrhea. No cultures were made in their case.

(b) Girl, 11 years old. Suspected rape. Profuse purulent vaginal discharge showing a large number of extracellular and fewer intracellular gram-negative diplococci resembling gonococci. Cultures were made on 4 slants of +0.5 Martin's pleuritic medium. Three of these tubes were kept at partial tension and the fourth aerobic. After 24 hours at 37 C. all the partial-tension tubes showed hundreds of minute translucent colonies which when subsequently studied seemed to correspond with the gonococcus. The aerobic tube, after 24 hours, gave 8 translucent colonies of gram-negative cocci which resembled the gonococcus. When this aerobic culture was 24 hours old, aerobic and partial-tension subcultures were made from a single isolated colony. The aerobic subculture showed luxuriant growth, while that at partial tension showed only a few scattered colonies. Microscopic examination of these subcultures revealed apparently pure cultures of gram-negative diplococci.

(c) Man, 26 years old. Subacute gonorrhea of some months' standing. Moderate urethral discharge obtained by expression. Direct smears showed numerous cocci resembling gonococci. Three slants of +0.5 Martin's pleuritic medium were inoculated, each with a loopful of pus. Two were kept at partial tension and the third aerobic.

The two former showed hundreds of typical translucent colonies composed of gram-negative cocci; the aerobic culture yielded 5 opaque colonies and 4 translucent colonies, which were not examined.

#### A PARTIAL-TENSION CLOSTRIDIUM

We encountered this organism first in glucose-broth blood cultures from a case of epilepsy. It forms spores which resist flowing steam for 15 minutes on 3 successive days or autoclaving at 15 pounds' pressure for some time under 30 minutes, but is killed at this temperature in 30 minutes. Dr. M. B. Cohen in this laboratory isolated it from glucose broth which was supposed to have been autoclaved at 15 pounds for 30 minutes, but in a special series of experiments in which he operated the autoclave himself, it was found not to resist this temperature. It has never been encountered in broth cultures without the glucose and a special series of experiments made by Dr. Cohen proved that the spores are present in glucose. Spores of both aerobic and partial-tension strains exist. The aerobic strains grow freely on incubation of the broth. But other tubes or flasks which appear sterile on aerobic incubation may contain the partial-tension spores and these are induced to grow with difficulty. If sterile blood is added to such a flask of glucose broth and then it is inoculated with *Staphylococcus aureus*, the spores apparently germinate slowly and after a week or so, the rods may be found in rather moderate numbers in the sediment but not in the supernatant fluid. Subcultures from this sediment usually yield the staphylococcus only, but occasionally after several days' incubation a growth of the clostridium will appear in the staphylococcus growth. When such a growth (on human-blood agar) containing spores is separated from the staphylococci by heating to 80 C. and planting in a deep tube of +0.5 Martin's medium containing pleuritic fluid, the clostridium develops slowly as a hazy line just beneath the surface of the medium. Subcultures from this line of growth on slants of the same medium yield no growth aerobically but luxuriant growth at partial tension. However, subcultures into another deep tube yield a similar growth just beneath the surface. The partial-tension slant culture remains true to type through several subcultures on Martin's pleuritic medium.

An aerobic strain was derived from it as follows: It was noted that transplanting from the hazy layer just beneath the surface of a deep shake culture would yield no aerobic growth; but if such a tube, the surface of which had been broken by the needle, was incubated for a

day or two longer, growth appeared on its surface and this could then be transplanted aerobically.

The partial-tension strain kept on Martin's pleuritic medium, differs in several points from the naturally or artificially derived aerobic strains. It forms longer and thicker rods, which often give the granulo-se reaction with Gram's iodine solution, and spore-formation does not take place until days have elapsed; altho spore-formation appears within 24 hours at 37 C. in partial-tension subcultures on +1 human-blood agar, as it does also in any culture of the aerobic strain.

#### A PARTIAL-TENSION BACTERIUM FROM A HUMAN KNEE JOINT

The patient was pregnant, had a purulent discharge from the vagina containing gram-negative diplococci resembling gonococci, and developed an arthritis of the left knee joint. This was aspirated and the sediment from the rather clear fluid was planted on +0.5 Martin's pleuritic medium and incubated aerobically. No growth. About 10 days later one of us superintended the collection of a second sample. This was markedly purulent. No bacteria could be found microscopically tho a number of staining methods were employed. The centrifuged sediment was planted on +0.5 Martin's pleuritic-agar slants and +1 human-blood-agar slants and incubated under aerobic and anaerobic conditions. These all remained sterile. Two plate cultures were made from the sediment in +0.5 Martin's pleuritic medium; one was incubated aerobically and the other at partial tension. The aerobic plate showed no growth, while thousands of very minute colonies appeared throughout the partial-tension plate. The organism proved to be a minute rod, which morphologically and culturally is much like *B. abortus*. We expect to make a further comparative study of this organism.

#### PARTIAL-TENSION CULTURES OF LEPTOTHRIX INNOMINATA

The organism was isolated by making anaerobic plates with material scraped from the human gum line. The anaerobic culture yields partial-tension subcultures but it will not grow aerobically. In deep-tube shake cultures it grows well throughout the medium and almost to its surface but not on the surface. As there is some doubt about this organism's having been cultivated before, we shall report its characters in detail separately.<sup>16</sup>

<sup>16</sup> Jour. Infect. Dis., 1916, 19, p. 299.

PARTIAL-TENSION CULTURES OF *BACILLUS TYPHOSUS*

The following single experiment suggests that the vast majority of typhoid bacilli growing in the body tissues are well over on the partial-tension side, and indicates for the first time, as far as we are aware, the extreme grade which the septicemia may attain in this disease.

Girl, ill with continued fever for 7 days. Rose spots on abdomen. Eight cubic centimeters of blood drawn from vein. Four large bottles of +1 broth inoculated with 1 c.c. of blood each. The rest of the blood was discharged into sterile 1.5% sodium-citrate solution. This last was centrifugated at high speed for about 10 minutes and the sediment hemolyzed with sterile distilled water and centrifugated again for one-half hour. Temperature about 25 C. (We realize that in making any accurate numerical count of the number of bacilli, the blood should have been chilled thoroughly between these operations. But in spite of the fact that the blood, after withdrawal, was at about 25 C. for nearly 1 hour before it was plated, we hardly think that this amount of incubation will account for the enormous numbers found. Further, we fail here to account for the very large number of bacilli which were present in the citrate plasma and which grew when this was placed in broth and deep tubes of agar.) The sediment was planted in several deep tubes of Martin's medium, in +1 broth, and a quantity which represented about 1.5 c.c. of blood was plated in each of 2 six-inch plates containing +1 Martin's medium and pleuritic fluid. One of these plates was incubated aerobically and the other kept at partial tension.

The aerobic plate showed thousands of colonies, but in the partial-tension plate they were present probably by the millions—in fact, they were so crowded that it was hopeless to attempt to count them. This partial-tension plate became contaminated after 48 hours by *B. subtilis*, which grew up one side of the plate and over its surface.

A partial-tension strain of *B. typhosus* was isolated as follows:

Some of the washed sediment of the patient's blood had been incubated aerobically in +1 broth for 48 hours at 37 C. There was a slight scum on the surface composed of apparently a pure culture of actively motile gram-negative bacilli. A little of the growth near the bottom of this tube was removed with a sterile capillary pipet and diluted with sterile salt solution. This dilution was then smeared with a loop on slants of +0.3 and +1 Martin's pleuritic medium. One-half of these were incubated aerobically and the rest kept at partial tension.

In 24 hours the aerobic slants showed numerous isolated colonies of a fairly uniform size (1-1.5 mm.). The partial-tension slants showed equally numerous 1-1.5 mm. colonies, but between these were almost as many pinpoint-sized colonies. Subcultures from these very minute colonies showed that they were of the partial-tension strain. The subcultures were made from the +0.3 Martin's pleuritic slants to

tubes of the same medium. For example: (a) In aerobic and partial-tension subcultures from the same 1-mm. colony on the partial-tension slant the growth was rather much alike in the two tubes with perhaps more growth on the aerobic. (b) Some of the minute pinpoint colonies on the partial-tension culture were mixed together and aerobic, partial-tension, and anaerobic subcultures made. The aerobic subculture yielded a delicate layer of growth, while the partial-tension subculture was covered by a luxuriant confluent layer.

The anaerobic subculture showed discrete, well isolated colonies—just as if all the bacilli in the minute partial-tension colonies were not fitted for anaerobic growth.

Litmus-lactose-agar plates made from the original blood-sediment-broth culture, from which these strains were isolated, yielded apparently a pure culture of typhoid-like colonies. It is perhaps a matter of chance here, but the vast majority of these plate colonies were deep colonies. A culture from one of these colonies fermented dextrose, levulose, galactose, maltose, and mannite with acid-production but no gas, and did not attack lactose or saccharose. This test was made on +1 Martin's pleuritic medium. The sugars, etc., were sterilized for 20 minutes at 15 pounds' pressure and added to the medium aseptically just before solidifying, as recommended by Martin.

The partial-tension strain was a very actively motile bacillus resembling the aerobic strain. When grown at partial tension on the same batch of sugar media used for testing the aerobic strain, it did not attack the afore-mentioned carbohydrates and alcohols during 4 days' observation. In these partial-tension cultures the formation of many filamentous forms during 24 hours' growth was a noteworthy observation, as we believe that this explains the formation of filaments in fluid cultures. We have also noted the tendency to formation of filaments in 24-hour-old colonies of *B. coli* on +0.5 Martin's pleuritic medium. The gross appearance of such colonies is not unlike that presented by the colonies of *Leptothrix innominata*.

#### DISCUSSION

It would seem probable that the majority of bacteria actively multiplying within the tissues of a host are adapted to a pressure of less than 21% oxygen. When transplanted from the tissues to artificial media under aerobic conditions, only a few bacteria, which are favored either by situation or by associated micro-organisms, become adapted to aerobic growth. However, as we have seen, when such bacteria are

brought out under conditions of reduced oxygen tension, the majority thrive well. What effect this observation may have on the cultivation of some of the hitherto unrecognized infectious agents remains to be seen. Such partial-tension parasites, when cultivated on such a medium as the sodium-phosphate pleuritic agar of Martin, become adapted to, or tenaciously cling to, the type of respiration required on this medium. They are with difficulty led over to the aerobic type of respiration by transplants on the same medium. If they grow at all under what appears to be aerobic conditions, close examination of the growth will show that they are not growing on the surface of the slant but within the surface layer. Further aerobic subcultures on the same medium have failed in our hands as a rule. In fact we have found that it is advisable to choose a medium of entirely different composition when one wishes to get an aerobic strain from a partial-tension one; for example, one may transplant aerobically to beef-extract agar or to human-blood agar or to maltose agar successfully, tho in the first place these media were unsuitable for the primary partial-tension growth from the tissues of the host.

The fact that the partial-tension strains of the bacillus from the knee joint and that of *B. typhosus* failed to attack glucose under partial tension, but attacked it vigorously when grown aerobically on the same medium, is certainly worth considering a little further. In the past, the tendency among medical bacteriologists has been to grow aerobically mass cultures of micro-organisms isolated from disease processes in the body, and to imply that the metabolic activities of such bacteria are comparable to those in the body. Theoretically it seems to us to be probable that the majority of micro-organisms thriving within the body exist under partial-oxygen tension and that some become very definitely adapted to such lowered oxygen concentration. Is it not possible that the partial-tension mode of nutrition in vitro is more nearly that which the micro-organism follows in the body of the host? If so, does failure to take this into consideration account for our inability in the past to recognize toxin-production by many species in vitro? Does this mode of nutrition lead to the building up of a bacterial cell body which has a very different chemical composition from the ordinary aerobic and anaerobic strains we have worked with in the past? The work of Bordet<sup>17</sup> on the antigenic properties of old and recently isolated strains of the whooping cough bacillus; the greater immunizing value of a "body strain" of *B. pestis* as shown by Row-

<sup>17</sup> Centralbl. f. Bakteriol., I, O., 1912, 66, p. 276.

land,<sup>18</sup> and the numerous recent observations by Rosenow on the tendency of certain strains of bacteria to localize at certain sites in the body, all point to the great importance of a closer study of the nutritional requirements of parasitic bacteria. Certainly past experience points clearly to the supreme influence of oxygen tension and temperature upon variation. Beijerinck<sup>19</sup> has shown that aerobic, partial-tension, and anaerobic strains of lactic-acid ferments may be derived by successive transfer from the top, middle portion, or bottom of fermented milk to corresponding regions of oxygen tension in the inoculated material. He points out very clearly that the character of the fermentation, that is, the end products, is controlled chiefly by temperature and oxygen pressure. It is unnecessary to quote in detail his numerous observations on variation but we are tempted to quote the following: "Variations in oxygen pressure above or below that most favorable to vital functions is undoubtedly one of the chief factors and these ferments only continue to display constant specific characters when continuously cultivated at a certain oxygen pressure—otherwise these characters disappear and in fact, or apparently, new ones originate. In wholly different groups of bacteria corresponding facts may be observed, hence their fundamental significance." Again, "That temperature is a decisive factor in the production of variants is shown by experience, for, prolonged cultivation above the optimum temperature gives rise to forms differing from the original stock."

<sup>18</sup> Jour. Hyg., 1914, 13, Suppl. 3, p. 403.

<sup>19</sup> Proc. Roy. Acad., 1907, 10, p. 17.

## LEPTOTHRIX INNOMINATA (MILLER)\*

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*Leptothrix innominata*, so commonly found in the "materia alba" along the human gingival margin, is apparently a strict parasite. That

is, its nourishment is largely dependent on the presence of certain proteins in the human body. According to Goadby<sup>1</sup> and Küster,<sup>2</sup> it has never been cultivated. We have isolated it in pure culture only once. This was due not to the difficulty of isolation so much as to the fact that at this time we became interested in other species. For the isolation of parasitic species we have been using Martin's medium<sup>3</sup> with reactions varying from +0.5 to +2, the medium containing an equal quantity

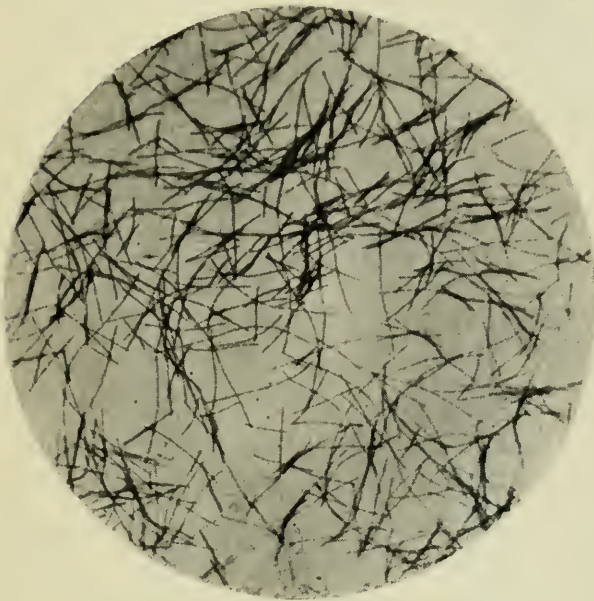


Fig. 1. *Leptothrix innominata* from anaerobic + 0.5 Martin's pleuritic slant. Methyl alcohol, dilute carbolfuchsin.  $\times 500$ .

of ascites or pleuritic fluid that had been heated to 56 C. for 1 hour on 5 or 6 successive days. Our work shows that this organism is adapted to a wide range of oxygen tension. It is a microaerophile-anaerobe; but we have not succeeded in obtaining an aerobic strain from it. This adaptation is what one might expect when one considers its natural habitat. When once grown away from the human tissues, it can thrive on beef-extract agar provided some suitable carbohydrate is present. Its marked action on the primary and secondary

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<sup>1</sup> The Mycology of the Mouth, 1903.

<sup>2</sup> Kolle and Wassermann's Handb. d. pathogen. Mikroorganismen, 1913, 6, p. 441.

<sup>3</sup> Jour. Path. and Bacteriol., 1911, 15, p. 76.



Fig. 2. Same as Fig. 1.  $\times 1000$ .

carbohydrates is noteworthy. Occasionally leptothrices which give the granulose reaction are encountered in the human mouth, tho the vast majority stain yellow with Gram's iodine solution. In the presence of maltose a few *Leptothrix innominata* also give the granulose reaction. This speaks for a unity of species.

#### MORPHOLOGY

The organism is rarely seen as a single cell but is commonly encountered in twos, and threes, and chains of 7 or more segments. Each segment is an elongated rod, usually with rounded ends tho frequently terminal segments may exhibit a tapering, somewhat pointed free end (Figs. 1, 2, and 3). When measured with a filar micrometer under the Zeiss 3 mm., N. A. 1.40, the cells in fresh unstained condition are 1.4 microns in diameter, but when fixed with methyl alcohol and stained with dilute carbolfuchsin, they are 0.84 micron in diameter. Single segments vary from 5 to 14 microns or more. Thus: 3 segments measured 26.5 microns, 7 segments measured 45.36 microns. The segments are usually attached to one another in a straight line and seem to be covered by a common delicate envelope. Long chains of segments—as in the milk culture, 336 microns long—bend like a hair and not like a chain of loosely joined rods.

The cytoplasm of the segments is fairly homogeneous but often shows a fine vacuolization along the central longitudinal axis. In cultures a week old on media containing fermentable carbohydrates, the cytoplasm is markedly vacuolated and the stained cells show many small irregularly distributed and deeply stained areas. No bodies resembling spores were seen. Nor were there any signs of motility in sealed and unsealed preparations.

In an anaerobic culture on +1 maltose agar, grown for 8 days at 37 C., a number of coiled forms were encountered. These apparently

coiled like a corkscrew at one end (Fig. 4) and then bent over and grew around the lengthening filament, thus resembling a twisted loop of rope (Fig. 5). These forms did not stain well and yet they appeared normal in the fresh preparations.

In scrapings from the teeth we have encountered forms showing a terminal pyriform swelling and such forms were found in pleuritic-fluid potato-water cultures, where also there were present fewer free ovoid and pyriform bodies (Fig. 6). These have about the same refractive index as the vegetative cells.

#### STAINING REACTIONS

Smears from cultures fixed by heat or methyl alcohol, stain readily with dilute carbolfuchsin or with Bordet's carbol toluidin blue or with Nicolle's carbol gentian violet. There is rather faint staining with Löffler's methylene blue in 5 minutes. The leptothrix loses the stain on treatment by Gram's method.

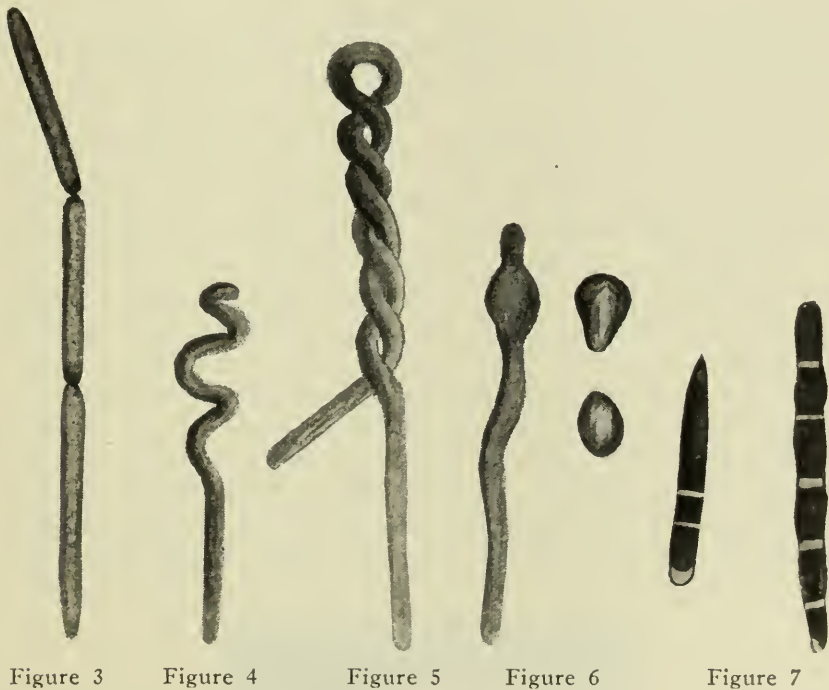


Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Fig. 3. Drawing of a fresh preparation from a colony on + 0.5 Martin's pleuritic medium, 48 hours at 37 C.

Fig. 4. Drawing of beginning looped form, from a fresh preparation from an 8-day + 1 maltose-agar anaerobic culture.

Fig. 5. Completion of looped form. Same source as Fig. 4.

Fig. 6. Swollen and pyriform and ovoid bodies from anaerobic pleuritic-fluid potato-water culture.

Fig. 7. Granulose reaction given by a few organisms in an anaerobic + 1 maltose-agar culture grown for 6 days at 37 C.

*Granulose Reaction.*—Most of the cultures were mounted in Gram's iodine solution and throughout the organisms were stained yellow except those from the +1 maltose anaerobic culture kept for 6 days at 37 C. Here a few, probably less than 1%, gave a marked reaction, the granulose being present in large blocks with intervening colorless segments (Fig. 7).

*Isolation and Biochemical Characters.*—Scrapings from the line of the human gum were suspended in sterile 0.85% salt solution, centrifugated, and resuspended 3 times. In this way a large number of the smaller associated bacteria were removed. The sediment was then spread on the surface of the medium, which had been solidified in Petri dishes. Various modifications of Martin's medium were used but the best result was obtained with the following: Martin's medium containing 1% of sodium sulfite and almost an equal volume of sterile human pleuritic fluid (heated to 56 C. for 5 successive days). The plates were kept at 37 C. under anaerobic conditions by means of pyrogallie acid and caustic soda, according to the plan suggested by Lentz.<sup>4</sup> The colonies first appeared as a loose thatch-like mass, which grew and became visible to the naked eye in from 4 to 5 days. When transplanted to slants of the same medium, the growth was much more luxuriant, isolated colonies reaching a diameter of from 0.5 to 3 mm. in a few days. Isolated colonies presented a fairly characteristic appearance when viewed through a hand lens; that is, they appeared like Medusa-lock colonies and as the irregularities in the thickness of different wavy portions of the colonies refracted the light, a pearly iridescence was exhibited (Fig. 8). This kind of colony, however, is not peculiar to the leptothrix, but may be shown by other bacteria which form threads; for example, *B. coli* and *B. typhosus* grown under partial tension.

Transplants to plain Martin's pleuritic medium grew equally well under anaerobic conditions and under partial oxygen tension produced by means of *B. subtilis*. Subcultures from the anaerobic and partial-tension cultures to aerobic slants of the same medium repeatedly remained sterile. The organism was planted on the following media and kept under anaerobic conditions with results as indicated. Growth fairly luxuriant on +1 maltose agar, Löffler's blood serum, +1 glucose agar containing blood—where the medium as well as the growth turned to a brownish-red hue—and on +1 lactose agar. No growth on +1 agar, +1 dextrose agar, +1 glycerin agar, +1 broth, +1 dextrose broth, +1 Dunham's peptone solution made from Liebig's extract. In litmus milk growth took place with the production of acid without coagulation, and the formation of a yellowish waxy mass which floated on the sur-

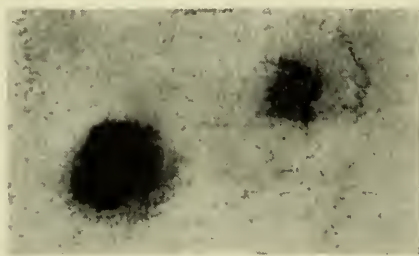


Fig. 8. Colonies of *Leptothrix innominata*. Four days' growth at 37 C., anaerobic, in + 0.5 Martin's pleuritic medium.  $\times 6$ .

face and resembled cerumen from the external ear. Microscopically this was composed entirely of coarse and fine fatty-acid crystals. The leptothrices were found only in the sediment, where they presented the usual appearance and breadth but many filaments—one, 336 microns long, was measured with the filar micrometer. These filaments contained irregularly placed spherical refractile bodies of the same diameter as the filaments themselves. They did not give the granulose reaction.

There was no growth on plain potato, but in a potato culture to which pleuritic fluid had been added, a flocculent growth appeared in the surrounding fluid. This was composed of normal forms, but many filaments showed irregular globular swellings and some free ovoid globular forms 3.3 microns in diameter. None of these forms gave the granulose reaction (Fig. 6).

*Fermentation Reactions.*—These tests were made according to the method recommended by Martin;<sup>4</sup> that is, to + 0.5 Martin's medium in tubes, there were added a few drops of a 10% carbohydrate or alcohol solution in distilled water (previously sterilized at 15 pounds for 15 minutes), and sufficient sterile litmus and pleuritic fluid. On such anaerobic slants there was marked acid-production with precipitation of serum in dextrose, levulose, galactose, lactose, saccharose, maltose, inulin, dextrin, raffinose, and mannose; mannite, erythrite, dulcitate, and isodulcitate were not attacked.

<sup>4</sup> Centralbl. f. Bakteriol., I, O., 1910, 53, p. 358.

## REMARKS ON THE FILTERABILITY OF BACILLUS BRONCHISEPTICUS \*

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Like many other micro-organisms *Bacillus bronchisepticus* has evidently been discovered and named many times. Theobald Smith<sup>1</sup> showed that some of Torrey's strains were identical with a bacillus causing an epidemic form of pneumonia in guinea-pigs, a disease which he had studied for some years. He also pointed out that very similar or identical bacilli had been described by previous workers. Interest in its comparative study was aroused by the work of McGowan, Ferry, and Torrey, which pointed to the possibility of its being the cause of canine distemper.

Ferry<sup>2</sup> made experiments to determine whether or not the passage of *B. bronchisepticus* could be brought into line with the claims of Carré that the virus of distemper is filterable. He goes into very satisfactory detail concerning the method of testing the filters he used (method of Bulloch and Craw), but unfortunately does not give the details of his filtration experiments. However, it is evident that 1 Berkefeld N. candle and 6 Pasteur F. candles were used, and that the filtration was conducted at room temperature during 1 hour and at 3 pressures, gravity 15 pounds negative and 225 pounds positive. He does not state at which of these pressures nor with which filters the positive results were obtained.

Since Theobald Smith<sup>1</sup> has demonstrated the identity of the bacilli mentioned, it is only fair to call attention to the fact that he had casually noted that the bacillus of guinea-pig pneumonia passed the small Berkefeld filter, and had asked me to go over and test his observations more thoroughly. This was done in a series of experiments.<sup>3</sup> In 2 fractional filtration experiments with the small Berkefeld No. 5, from 75 to 80 c.c. of filtrate came over before any bacilli passed the filter—thus was proved the absence of any gross leakage. The same filters did not allow *B. coli* to pass.

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<sup>1</sup> Jour. Med. Research, 1913, 29, p. 291.

<sup>2</sup> Jour. Path. and Bacteriol., 1915, 19, p. 488.

<sup>3</sup> Wherry: Jour. Med. Research, 1902, 8, p. 322.

There is no doubt, however, that many of the Berkefeld filters show leakage. I tested 12 new candles a few years ago and found that only one held back *B. pyocyaneus* from the first portions of the filtrate. Contrary to what is expected from some of the observations of Bulloch and Craw, it has been my experience that an efficient candle may be cleaned by boiling in sodium-carbonate solution and resterilized by autoclaving a number of times without impairing its efficiency for such an organism as *B. pyocyaneus* in broth cultures.

# A NEW MODEL OF DOUBLE PIPET HOLDER AND THE TECHNIC FOR THE ISOLATION OF LIVING ORGANISMS \*

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The working parts of the old models of double pipet holders are made of stock brass. In the new model to be described here they are made of cast phosphor bronze. The male and the female milling of the old models are cut in the working parts; the male milling of the new model is cut separately and fastened to the working part with screws. In the old models after a short period of use, lost motion develops in the milling, destroying the accuracy of the instrument. Also, when the working part is moved upward, the male milling immediately passes out of its bearing; in the new model the male milling is never disengaged. A space of  $5/1000$  inch is left between the working part and the face of the milling, and in this space there are 5 brass shims, each of which is  $1/1000$  inch in thickness. Thus if the bearings after a time become loose, the trouble is quickly remedied by unscrewing the screws and removing the working part and one of the thin sheets of brass, then replacing the working part and gradually tightening the screws until the working part is easily operated with the mill-head. In the older models, the bearings of the transverse carriages are too short, so that when they move outward from the center of the instrument, they develop a marked looseness. In the new model, this defect has been entirely overcome. An arm projects at right angles to the perpendicular carriage (Fig. 1); on this arm is a carriage traveling at right angles to the perpendicular carriage. On the upper surface of this carriage there is another carriage which travels at right angles to the one supporting it, and on the upper surface of this carriage an arm projects towards the center of the instrument. This is the pipet holder.

The carriage has the female milling cut on one of its surfaces, while on the opposite surface of this part there is a clamp which fits a small metal block extending from the side of the stage of the microscope. When the mill-head 1d is tightened, a metal clamp is moved upward to engage the block projecting from the side of the stage of the microscope, thus making the instrument secure and rigid. The male milling 1B is fastened to a face plate, which engages the female milling, and to this face plate the perpendicular carriages are made fast with screws; the transverse carriage is operated by the

\* Received for publication February 25, 1916.

millhead 1A, which when turned moves the transverse carriage backward and forward, and by this movement the perpendicular carriages are moved forward and backward. The advantage of the position of the millhead in this model, as compared with the older models, is that one does not have to reach around in front of the microscope to operate this carriage. At the opposite end of the screw operated by the millhead 1A, is a small knurled screw 1C. This screw when tightened, takes out all the lost motion in the screw which operates the carriage. Thus not only can the working parts be adjusted by the removal of the shims, but the screws likewise can be adjusted when they become loose

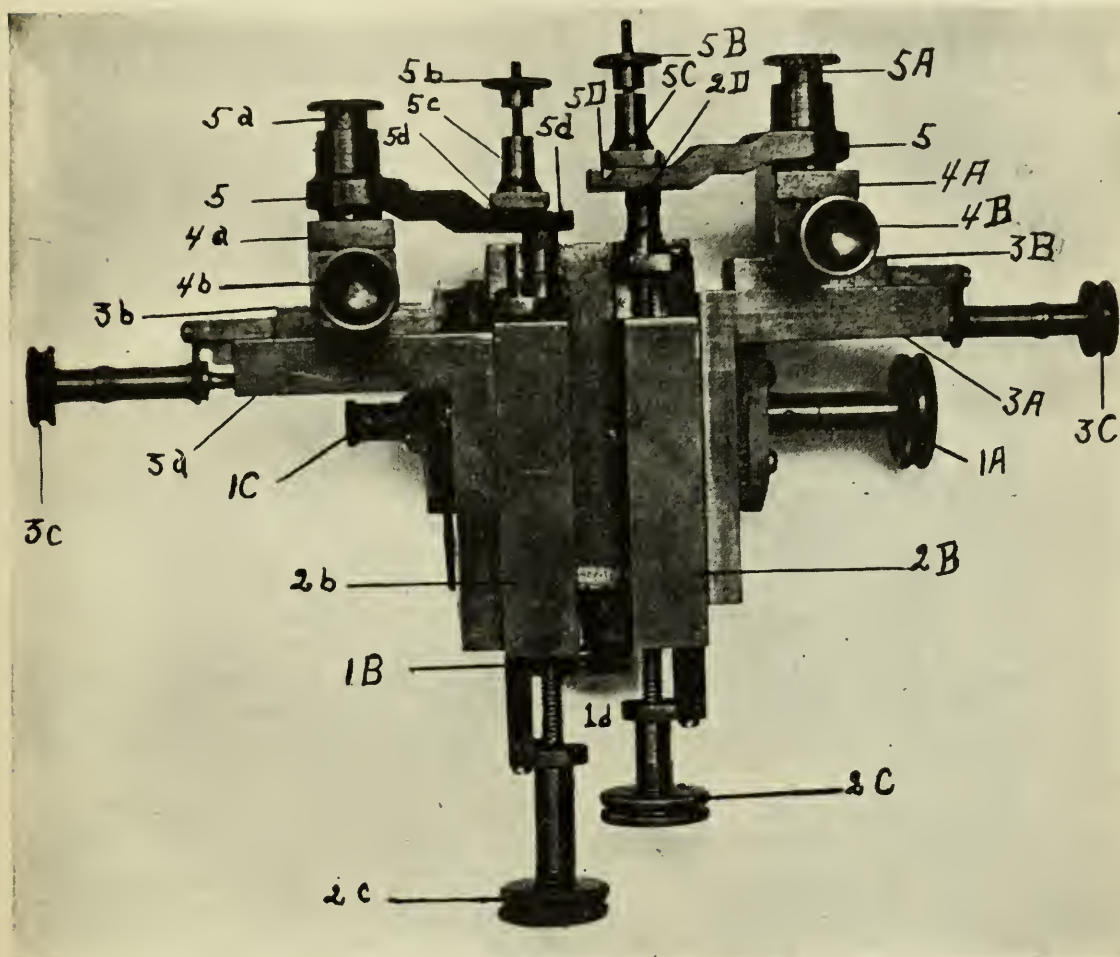


Fig. 1. The new model of double pipet holder.

in their bearings. The perpendicular carriages 2B, 2b are operated by the millheads 2C, 2c. These millheads when turned, cause the carriages to move up or down. At the opposite ends of the screws operated by the millheads 2C, 2c are small knurled thumb screws of the same type as 1C. These, when tightened, adjust the lost motion developed by the operating screws in their bearings. Extending at right angles to the perpendicular carriages is an arm which is part of each of the carriages 2B, 2b, and is marked 3A, 3a. On the upper surface of these arms are the carriages 3B, 3b, operated by the mill-

heads 3C, 3c. When the millheads 3C, 3c are turned, these carriages move at right angles to the perpendicular carriages. On the upper surfaces of the carriages 3B, 3b are the carriages 4A, 4a, operated by the millheads 4B, 4b and moving at right angles to 3B, 3b. On the upper surfaces of these carriages are the clamps for the pipets proper 5, 5, and the bearings of the clamps. If the thumb screws 5A, 5a are loosened, the clamp can be moved to any desired angle, and the thumb screws 5A, 5a again tightened to make the angle of the pipet secure. At the opposite end of the arm of the pipet clamp to the bearing are other thumb screws 5B, 5b, which, when operated, press on the plate clamps 5C, 5c fitting over the grooves 5D, 5d; by this operation the pipet is held securely in the grooves 5D, 5d.

The two pipets can be moved forward and backward by turning the millhead 1A. The perpendicular carriages can be moved up and down independently of each other by turning the millheads 2C, 2c. On the upper surface of the arm of the perpendicular carriages are other carriages, which are moved at right angles to the perpendicular carriages by the millheads 3C, 3c, and on the upper surfaces of these carriages is another set of carriages, which are moved at right angles to the carriages 3B, 3b by the millheads 4B, 4b, and on the upper surface of these carriages is the long arm which can be turned at any desired angle by loosening the thumb screws 5A, 5a.

Having described the parts of the double pipet holder and their operation, I shall now explain the accessory apparatus, and the preparation of it for the isolation of living bacteria.

*Isolating Cell.*—The cells used for the isolation of living bacteria are of 2 types, glass cells and brass cells. Dr. Barber (of whose technic some of the steps here are modifications) uses the glass cell, while I use a brass cell. This brass cell measures 80 mm. by 27 mm. and the height of the cell from the glass window to the upper edge of its walls is 15 mm. No particular advantage is offered by the brass cell over the glass cell in the isolation of organisms, but the broad upper edges of the brass cell which come in contact with the cover slip, when coated with vaselin offer a more secure joint. In the bottom of the cell there is a glass window made secure and water-tight with gum balsam.

*The Preparation of the Isolating Cell.*—This is very simple. The lateral and back walls of the cell are covered with thin blotting or filter paper. This lining acts as an equalizer of the moisture contained within the cell and thus prevents the evaporation of the culture media or salt solution on the under surface of the cover slip. The bottom of the cell is next flooded with sterile-distilled water. The upper edges of the cell which come in contact with the cover are then thoroughly coated with vaselin. The vaselin holds the cover slip in position and lutes the point of contact of the cover slip with the edges of the cell, making an air-tight joint between them.

To protect the living organisms from a possibly fatal change of temperature in the transfer from the diseased part to the under surface of the cover slip I have devised an electrically heated jacket which, when placed around the isolating cell, maintains its temperature at 35.5-37.5 C. A further advantage of this jacket is that one does not have to hurry in the isolation of living organisms for fear of their dying before the transplantation from the under surface of the cover to the test tube or to the animal can be accomplished.

*Preparation of the Cover Slip.*—The cover slip used is of 2 sizes, 50 by 24 mm. and 40 by 24 mm. The cover slip is washed with soap and running water, rinsed in running warm water, and thoroughly dried with a piece of clean muslin. It is then given a coating of vaselin. Excess of vaselin is thoroughly removed from the cover slip with another clean piece of muslin, and then the surface is polished with Japanese bibulus paper. The vaselin on the surface of the cover slip prevents the fine droplets of condensation water from uniting to form large drops. After the cover slip has been prepared,

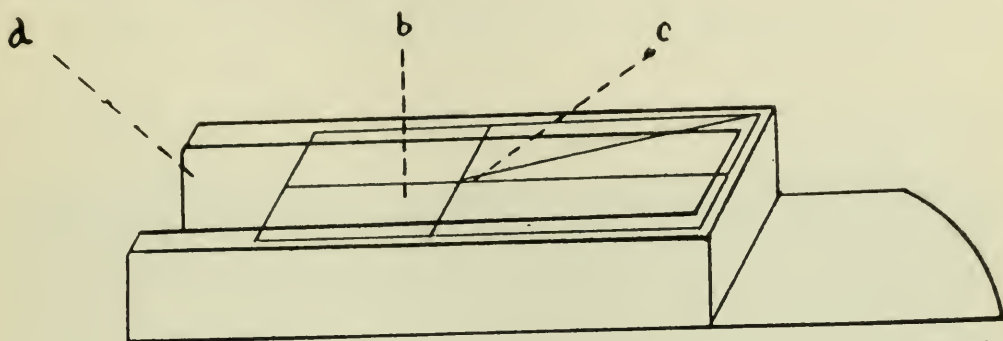


Fig. 2. (a) The isolating cell. (b) The ruled cover slip. (c) The ruled cover slip in position on the cell.

it is passed through a gas flame several times to insure sterilization. It is now placed on the cell.

Its upper surface is ruled as follows: A fine capillary pipet, drawn from a piece of glass tubing, is covered with thick India ink and the point placed at the outer edge of the cover slip. The instant that the pipet comes in contact with the cover slip the entire pipet is brought in contact with the surface of the cover slip. The point of the pipet is now drawn from the front of the

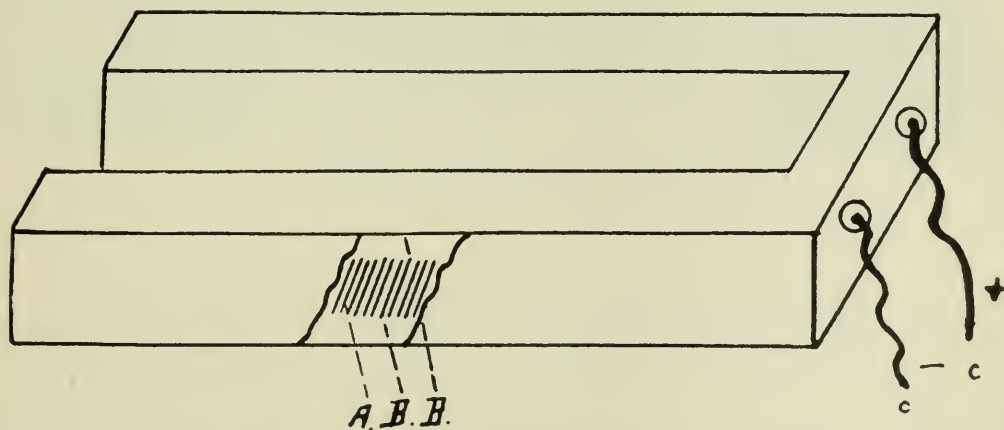


Fig. 3.—Electrically heated jacket for the isolating cell. A is the heating element, B, B is the asbestos insulation, and C, C the terminals of the heating element.

cell, backward; this movement leaves a black line on its surface. The pipet is again covered with India ink and another line made at right angles to the one passing through the longest diameter of the cover. A third line is drawn to make an angle of 95 degrees with each of the previous lines. The completed cover slip has 3 lines, none of which is parallel to another. The line

which is at 95 degrees to the other two lines, is used as a guide for the placing of the droplets containing the isolated bacteria.

*Placing the Droplets on the Under Surface of the Cover Slip.*—A pipet is drawn and the excess of the capillary portion over 10 to 15 cm. is broken off. From 10 to 15 mm. of the end of the pipet are bent at right angles to the pipet. A piece of rubber tubing is then attached to the opposite end. The free end of the rubber tubing is between the lips, and the point of the pipet is immersed in broth or sterile normal salt solution; by gentle suction the desired quantity of liquor is drawn into the pipet. Now the placing of a series of small droplets around a large central drop on the under surface of

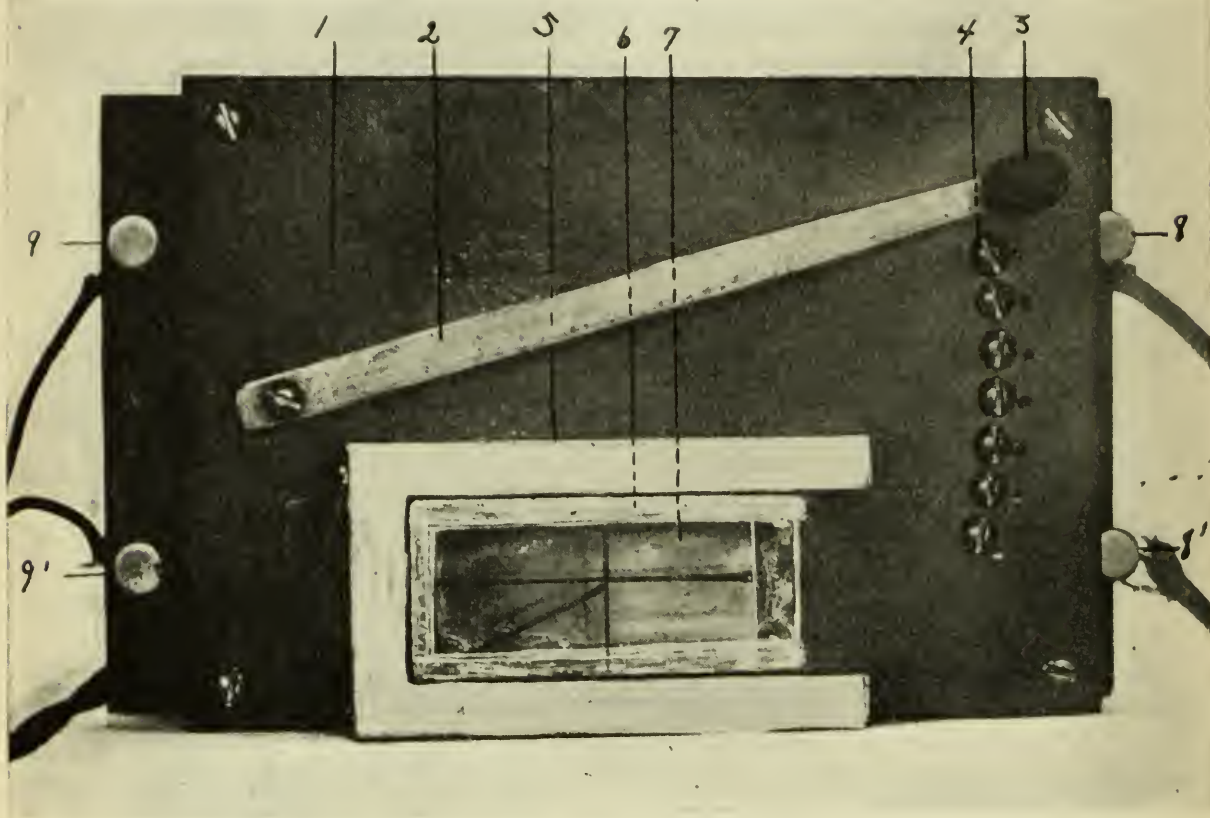


Fig. 4. Rheostat for controlling the temperature of the warm jacket and the cell. Beginning at 1, which is the rheostat proper, 2 indicates the arm of the rheostat, used for making the contact points; 3 is the handle of the lever; 4, the contact points which control the amount of electricity entering the coil in the warm jacket; 5, the warm jacket; 6, the isolating cell surrounded by the warm jacket; 7, the cover slip in position on the surface of the cell, and on its surface the ruling used; 8 and 8' are the two poles of the incoming electricity; and 9 and 9' are the outgoing poles, which are connected with the coil in the heating jacket. Any desired temperature can be obtained with this instrument around the cell.

the cover slip is accomplished with the point of the pipet. The object of the small droplets around the central drop is to insure the latter against rapid evaporation. A coarse capillary pipet is now made. It is charged with sterile distilled water, the point passed under the cover slip, and a series of small droplets made on the under surface of the cover slip at its outer edge. These droplets equalize the moisture in the cell and thus prevent the evaporation of

the droplets on the under surface of the cover which contain the emulsion of bacteria, and also the very small droplets which hold the isolated bacteria in suspension.

*The Inoculation of the Central Drop.*—A small sterile platinum loop is used for the collection of pus from the focus of suppuration, after which the loop is cautiously passed to the central drop, and its pus content thoroughly mixed into that drop. The mixing having been completed, the cell is placed under a bell glass jar or large Petri dish until the pipets have been made and charged.

*The Making of Capillary Pipets for Isolation Purposes.*—The apparatus consists of a pair of small thumb forceps and 2 Bunsen burners, one of which is remodeled in such a way as to offer a flame for making the very fine capillary pipets needed for the isolation of bacteria. The glass tubing used for making the capillary pipets has a caliber of 2 mm. and is cut into 14-cm. lengths. It is held by the thumb, index, and second fingers of the left hand

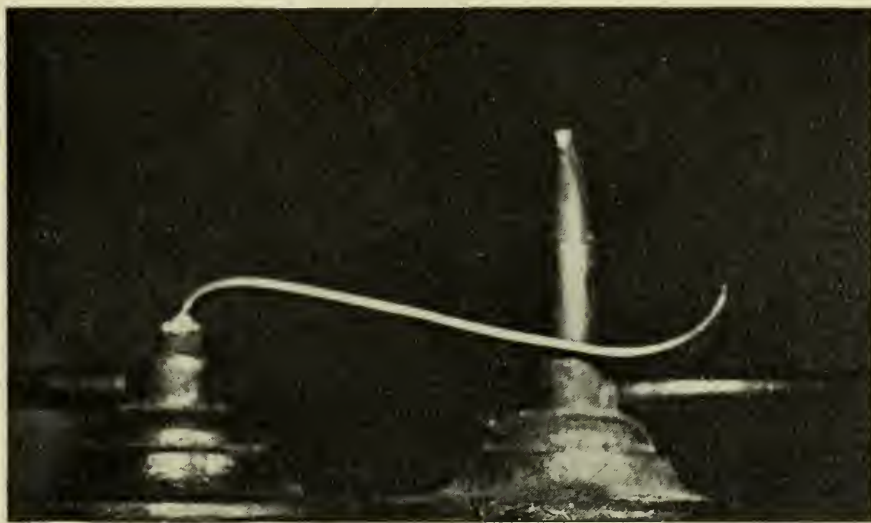


Fig. 5. The two types of Bunsen burners.

at a comfortable distance from the end of the pipet which is in the flame. The thumb forceps are held by the thumb, index, and second fingers of the right hand. As soon as the portion in the flame becomes soft enough to bend of its own weight, the end of the molten portion is grasped firmly with the thumb forceps, and at the same time the glass tubing is removed from the flame, and a lateral pull made from left to right, and continued until the desired caliber of pipet is drawn. The hands are then held in their relative positions for half a minute to allow the glass to cool. We now have, approximately, a straight pipet instead of a crooked one. The excess of glass of the capillary pipet over 85 mm. is broken off.

The very fine capillary pipets are harder to make than the coarse ones. The shank of the coarse capillary pipet is grasped with the thumb and fingers of the left hand while the free end of the pipet is grasped with the thumb forceps in the thumb and fingers of the right hand. The outer portion of the pipet which is near the thumb forceps is held in the fine flame, and the instant the glass becomes soft enough to bend, a lateral pull is made to the right if a moderately coarse pipet is wanted, and to the left if a very fine pipet is wanted.

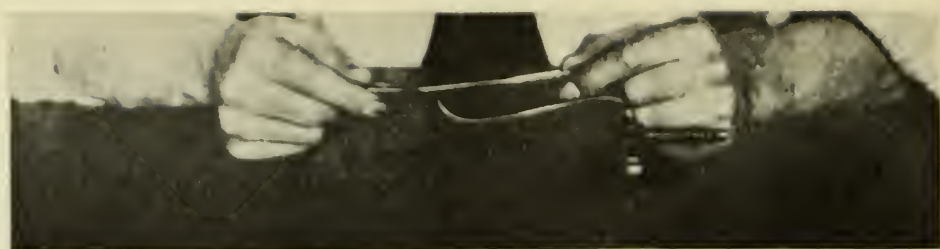


Fig. 6. The position of the hands for making the fine capillary pipet.

Now with the shank of the pipet grasped between the thumb and fingers of the left hand and the free end supported by the point of the forceps in the right hand, the pipet is held in the very fine flame while at the same time gentle pressure upwards is made with the forceps; as soon as one feels the glass giving, the pressure is slightly increased and at the same instant the pipet is removed from the flame.

If on examination of the pipet it is found that the angle is too acute or too obtuse, the defect must be corrected, for if it is not at right angles it can not be successfully used. To correct the angle of the pipet the shank is grasped with the thumb and fingers of the left hand and the point of the forceps is placed on the outer surface; the pipet is then held in the very fine gas flame and gentle upward pressure made with the forceps. As soon as the pipet commences to bend it is immediately removed from the flame and gentle upward pressure made with the forceps. If the angle is too acute, the point of the thumb forceps is placed on the inside of the pipet and it is again placed in the very fine flame, gentle outward pressure being made with the point of the thumb forceps. As soon as the glass commences to bend, the pressure is continued and at the same instant the pipet is removed from the flame. If the angle of the pipet is too obtuse, the point of the thumb forceps is placed on the outside of the fine capillary pipet and it is placed in the fine flame and the angle corrected by bending the pipet inward.

The next step is to break off the excess of the fine capillary pipet over 15 mm. with the points of the thumb forceps, which have been sterilized in the gas flame. The rubber tubing is now fastened to the pipet. The pipet is charged with sterile salt solution or broth by immersing the fine point in the liquor and making suction on the free end of the rubber hose held in the mouth, which draws the desired quantity of fluid into the pipet. After placing the cell on the mechanical stage, the pipet is centered.

*Centering the Point of the Pipet.*—The cell is moved to the right until one half of the field is occupied by the cover slip, and the end of the black line which passes through the longest diameter of the cover slip is in the center of the field. The tube of the microscope is then moved up out of the way and the pipet is placed in the groove of the clamp, 5D, 5d, of the pipet holder, and is pushed inward toward the black line until it is approximately opposite it. The field is then brought into focus, and the point of the pipet is sought by raising and lowering the tube of the microscope. If the pipet is out of the center of the field, it is centered by manipulating the millhead of the carriage which controls the movement in the correct direction. The first pipet having been centered, the second pipet is made by the same technic as was used for making the first. It is adjusted in the same manner also. In addition to

being centered the pipet must be so adjusted that Pipet 1 in moving up or down will in no wise interfere with Pipet 2 and vice versa. Both points should be approximately in the center of the field for this will facilitate the work.

#### THE ISOLATION OF LIVING BACTERIA

An area in the emulsion of the pus, rich in bacteria, having been found, the free end of the rubber hose attached to Pipet 1 is placed between the lips and the pipet gradually moved upward with Millhead 2c. As soon as the point of the pipet is in the same focus gentle suction with the mouth draws the desired quantity of the emulsion of pus into the pipet. The pipet is lowered out of focus with Millhead 2c. The mechanical stage is moved to the left of the line which is at right angles to the horizontal line, and to the space below the horizontal line on the under surface of the cover slip. The desired area having been selected the pipet is moved up with Millhead 2c until the point of the pipet is in focus on the under surface of the cover slip; by gentle pressure on the free end of the rubber hose held between the lips, with the mouth, the desired size of droplet is made. The pressure is then relieved and at the same instant the pipet is moved down out of focus by turning Millhead 2c. This process is continued until a series of small droplets are placed on the under surface of the cover slip. Pipet 1 is now moved out of the way and Pipet 2 brought into focus with Millhead 2 C. Small droplets made with Pipet 1 are further diluted with broth from Pipet 2. By this further dilution the bacteria are greatly separated from one another so that isolation becomes easier. This preliminary step is accomplished with a Leitz objective No. 4 and a Huyghenian eye piece No. 5. The isolation of the organism is done with Leitz objective No. 6 or 7 and Huyghenian eye piece No. 2.

The procedure for the isolation of a streptococcus, for example, in pus thus conveyed to the cell is as follows: As soon as a chain is found, the point of the pipet is brought into the same focus by turning Millhead 2C. Then gentle suction is made with the mouth on the free end of the rubber hose attached to the pipet. The instant that the chain of streptococci enters the pipet the suction is stopped and at the same instant the pipet is moved out of focus by turning Millhead 2C, thus preventing all other bacteria from entering the pipet. The mechanical stage is now moved to the left until the line which runs at 95 degrees to the other lines appears. The pipet is then gradually moved up until its point is in focus with one of the small droplets of condensation water which have collected on the under surface of the cover slip.

Gentle pressure with the mouth at once causes the small droplet to increase in size and as soon as the chain of streptococci appears, the pressure is relieved and the pipet instantly moved down out of focus with Millhead 2C. The same technic is followed in the isolation of any other micro-organism present. One soon becomes so expert that the dilution of the original droplet is not necessary, the isolation being made from the first droplet.

In making transplants a method which I find very good is as follows:

The organism having been isolated. Pipet 1 is removed from the pipet holder and a new pipet made and charged with the desired variety of broth and adjusted as previously described. The mechanical stage is now moved to the left, searching for the droplets containing the desired organism, which, when it is found, is drawn into the pipet. The stage is again moved and the organism is dispatched to the under surface of the cover slip, and again picked up with the pipet. The excess of broth is then drawn into the pipet from the droplet. The pipet is now removed from the holder and the broth is carefully drawn to a safe distance from the point of the pipet so that it can be sealed in a gas flame. Then, labeled with the date, hour, and the kind of organism, it is placed in the incubator.

- Another method for obtaining cultures of the isolated organisms is that used by Dr. Barber. The organism after isolation is placed in a droplet of culture medium on the under surface of a clean sterile cover slip. The cover slip is then transferred from the cell to a ground-glass concave slide. The preparation of the slide before making the transfer of the cover is as follows: The slide is washed in running hot water and sterilized with alcohol. The ground surface of the slide is then given a liberal coating of vaselin, and in the concave portion of the slide there is placed a small droplet of sterile distilled water. The cover is placed on this slide, the small droplet being on the inside of the concave slide. The slide is then labeled with the time, date, and kind of organism. This method has an advantage over the first method in that one is able to study the growth rate of the organism and at the same time commence an isolated culture. The technic for the study of the growth rate of the colon bacillus, which can be used for the study of other organisms also, has been worked out by Dr. Barber.<sup>1</sup>

The use of the new model of double pipet holder is in nowise confined to the bacteriologist; it can be used by cytologist, embryologist, zoologist, and chemist. I believe that with the double pipet holder many problems can be worked out with greater precision.

<sup>1</sup> Jour. Infect. Dis., 1908, 5, p. 379.

## COMPLEMENT-FIXATION IN TUBERCULOSIS \*

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The discovery of the tubercle bacillus by Koch in 1882 placed the diagnosis of tuberculosis on a substantial foundation, but the bacillus cannot always be demonstrated early in the discharges, frequently never appearing, and even if present giving little clue to the activity or inactivity of the disease. Clinical findings give us information regarding activity or inactivity only in a crude way. Thus far biologic methods of diagnosis have been of little practical value with one exception—complement-fixation. Tho not fulfilling the early expectations, this method of diagnosis is gradually being improved so that there is promise of its becoming as valuable a diagnostic test as the Wassermann in syphilis.

### HISTORICAL REVIEW

The first application of complement-fixation in tuberculosis was made by Widal and LeSourd<sup>1</sup> in 1901. They obtained deviation of complement in certain cases of tuberculosis, using as antigen homogeneous emulsions of tubercle bacilli of the Arloing-Courmont strain. In 1903 Bordet and Gengou<sup>2</sup> demonstrated the presence of antibody capable of uniting with tubercle bacilli and fixing complement in the sera of tuberculous animals. Wassermann and Brück<sup>3</sup> in 1906 demonstrated the presence of an antibody to tuberculin in patients treated with tuberculin, but they examined only 13 cases of pulmonary tuberculosis. Caulfield<sup>4</sup> in 1911 examined 104 cases of pulmonary tuberculosis with bacillary emulsion as antigen and obtained 33% Turban I cases, 70% Turban II, and 62% Turban III positive results. Laird<sup>5</sup> (1912) out of 84 tests in 34 cases obtained 24 positives in 4 cases, using watery emulsion of tubercle bacilli (which he does not describe); his results were inconclusive. Hammer,<sup>6</sup> using O. T. and extracted tuberculous nodules, obtained 97% positive results in 46 tuberculous cases. Calmette and Massol,<sup>7</sup> using preparations made from tubercle bacilli by extracting with water and peptone, obtained in 134 cases 92.5% fixation. Fraser<sup>8</sup> (1913), testing a large variety of antigens, found that living bacilli gave no fixation in 96.6% of normal individuals, but gave positive reactions in 42.3% of tuberculous individuals. She states that the most reliable antigen

\* Received for publication March 2, 1916.

<sup>1</sup> Cited by Shennan and Miller, *Edinburgh Med. Jour.*, 1913, 10, p. 81.

<sup>2</sup> *Compt. rend. Acad. de sc.*, 1903, 137, p. 351.

<sup>3</sup> *Deutsch. med. Wchnschr.*, 1906, 32, p. 449.

<sup>4</sup> *Jour. Med. Research*, 1911, 24, p. 122.

<sup>5</sup> *Ibid.*, 1912, 27, p. 163.

<sup>6</sup> *München. med. Wchnschr.*, 1912, 59, p. 1750.

<sup>7</sup> *Compt. rend. Soc. de biol.*, 1912, 73, p. 120.

<sup>8</sup> *Ztschr. f. Immunitätsf.*, 1913, 20, p. 291.

is prepared from living human bacilli, and that diagnostically the complement-fixation test with living bacilli is of more value from the standpoint of positive results than any other reaction discovered to date. She believes the absence of antibodies accounts for the low percentage of results obtained. Dudgeon, Meek, and Weir<sup>9</sup> also tested a large number of antigens, and in 102 untreated cases obtained 86 positive results, while all cases which had been treated with tuberculin gave positive results. Products of the bacilli themselves were found to be the most satisfactory as antigen. With an alcoholic antigen<sup>10</sup> prepared from tubercle bacilli they obtained from a total of 234 cases, 209 (89.3%) positives, 194 of these on 1st examination, 11 (of the 15 negative) on 2nd examination, and 4 more on 3rd examination. Besredka<sup>11</sup> (1913) prepared an antigen by growing tubercle bacilli (of a questionable nature since they grow in from 24 to 48 hours) on egg broth, heating it, and filtering. With this antigen Bronfenbrenner<sup>12</sup> (1914) obtained a very high percentage of positive results, 93.8% in active cases, and 55.5% in convalescents, while suspected cases gave 75% and syphilitic sera 24% positive reactions. Inman,<sup>13</sup> and Kuss, Leredde, and Rubenstein<sup>14</sup> found this antigen nonspecific. McIntosh, Fildes, and Radcliffe<sup>15</sup> (1914) also justly criticized Besredka's antigen and concluded, after testing a large number of antigens, that the living bacillary emulsion was best, yielding 76.7% positive results in 43 definite cases of phthisis, 80.7% in surgical tuberculosis, and 37.5% in glandular tuberculosis. Of 87 normal individuals only 3 gave positive reactions (2 of these were lepers and 1 had Addison's disease). Negative reactions were obtained in 18 syphilitic patients. They look upon a positive reaction as indicative of active tuberculosis. Stimson<sup>16</sup> (1915), who gives a fairly exhaustive table of the recent literature, reports a small number of cases, in which a variety of antigens were used, but his results were inconclusive. Craig<sup>17</sup> (1915) reports the results of examination of 166 cases of pulmonary tuberculosis, in which he employed as antigen an alcoholic extract of several strains of human tubercle bacilli which had been grown on a liquid medium of alkaline broth containing egg; 96.2% positive results were obtained in active cases and 66.1% positive in inactive cases. One hundred fifty cases of syphilis gave only 2 positive reactions and these on further examination revealed lesions in the lungs. One hundred other diseases examined all gave negative results. It remains, however, for future investigators to corroborate Craig's findings by proving this antigen to be specific.

The most reliable investigators concede that a suspension of living tubercle bacilli is the only one of the many antigens used, that is of specific value. The objections to the bacillary emulsion are the small leeway between the antigenic and the anticomplementary dose, the turbidity produced in the tubes, and the fairly high percentage of non-specific reactions. In the hope of overcoming these difficulties, it was

<sup>9</sup> Lancet, 1913, 184, p. 19.

<sup>10</sup> Jour. Hyg., 1914, 14, pp. 52, 72.

<sup>11</sup> Compt. rend. Acad. d. sc., 1913, 156, p. 1633.

<sup>12</sup> Arch. Int. Med., 1914, 14, p. 786.

<sup>13</sup> Compt. rend. Soc. de biol., 1914, 76, p. 251.

<sup>14</sup> Ibid., p. 244.

<sup>15</sup> Lancet, 1914, 185, p. 485.

<sup>16</sup> Bull. Hyg. Lab. U. S. P. H. and M.-H. S., 1915, No. 101, p. 7.

<sup>17</sup> Am. Jour. Med. Sc., 1915, 150, p. 781.

decided to try to obtain the antigen from the tubercle bacillus by processes as nearly identical with those that occur in the body as possible. With this in mind, and with the realization that bacterial antigens are probably of protein character, the following investigations were carried out.

To determine the most favorable condition for the liberation of the antigenic products from the tubercle bacillus, heavy suspensions of living tubercle bacilli of human origin were made in sterile tubes with sterile physiologic salt solution. One set of tubes was incubated, another set was kept at room temperature, and a third, as control, heated for 30 minutes in a boiling water bath to kill the bacilli, and then incubated. The disintegration of the bacilli was observed in the amount of noncoagulable nitrogen liberated, determined by the Folin micro method. A typical set of these results is plotted in Chart 1. (The complete experimental data, methods, and results will be reported in a subsequent paper.) As noted in Chart 1, there was a gradual liberation of

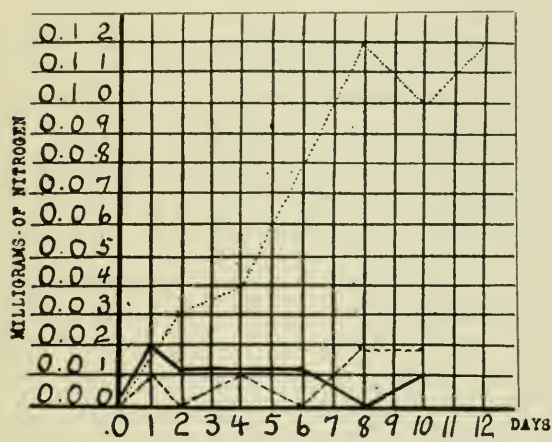


Chart 1

— The heated and incubated control.  
 ..... The incubated aseptically.  
 - - - - - The room-temperature aseptically.

noncoagulable nitrogenous substances from the tubercle bacillus at incubator temperature, reaching its maximum at about the 8th day. This did not take place after the bacilli had been killed by heat. At room temperature it did not occur to any appreciable extent within 10 days.

The process by which these nitrogenous materials appeared was next studied. It is of course conceivable that it might be either a simple dissolving out of endogenous nitrogenous materials from the bacilli, or the result of enzyme action, an autolysis. As shown by Wells and Corper<sup>18</sup> toluene destroys the tubercle bacilli but leaves the enzymes intact. Chart 2 gives the results of an aseptic (suspension of bacilli in salt solution with-

out the addition of an antiseptic) and antiseptic (toluene) experiment carried out at incubator temperature. Autolysis of tubercle bacilli was perceptible at the 2nd or 3rd day, and reached a maximum at the 6th or 8th day at incubator temperature. These experiments were repeated with bovine tubercle bacilli, and the same found to hold true.

The next question was whether or not this autolysis bears any relation to the increase of antigenic strength in the autolysate. In order to test this, the autolysate from a series of suspensions of tubercle bacilli in sterile physiologic salt solution was tested at definite intervals for noncoagulable nitrogen content, and coincidentally titrated for antigenic strength. Varying amounts of the antigen were titrated against a four-plus tuberculosis serum and the amount of complement-fixation noted. As seen from Table 1, altho the nitrogen figures and the antigenic titer do not show an exactly parallel increase, they do bear a certain relation to each other, and it is to be noted that an antigenic

<sup>18</sup> Jour. Infect. Dis., 1912, 11, p. 288.

titer of 0.1 c.c. on the 1st day became gradually and consistently a titer of 0.001 c.c. on the 6th day. Thus the autolysate from suspensions of living virulent tubercle bacilli grew stronger in antigenic titer coincidently with the occurrence of autolysis.

In order to test the value of the autolysate antigen it was compared in a large series of cases (over 600) with the bacillary emulsion. In a general way it can be stated that the autolysate antigen possesses the following advantages over the bacillary emulsion: it has a much larger range between the antigenic and anticomplementary doses (even 0.2 c.c. of a 0.001-c.c. strength has no anticomplementary effect); it does not lose its titer when kept on ice (several autolysates have kept their titers for 4 months); it produces no interfering turbidity in the hemolytic system; and it is more specific than the bacillary emulsion.

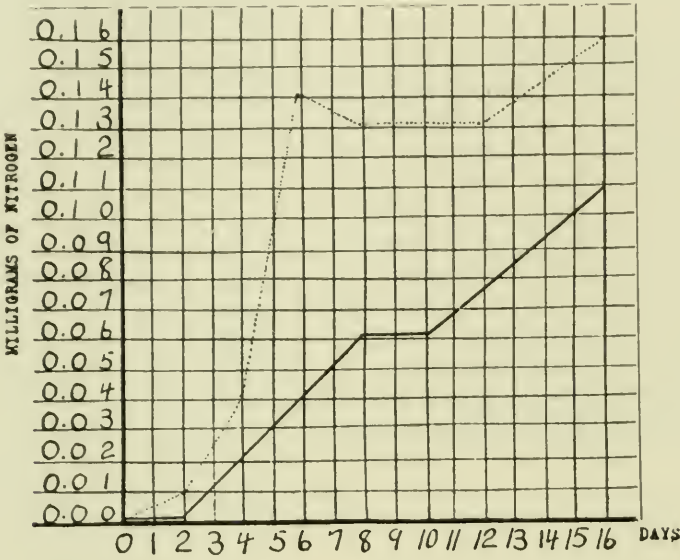


Chart 2  
——— Aseptic autolysis in incubator.  
..... Antiseptic (toluene) autolysis in incubator.

More than 600 examinations were made with the bacillary emulsion and the autolysate as antigens, but for the sake of accurate comparison only the results from 361 cases are given in tabulated form, only such cases being included as had been accurately worked up from the clinical standpoint. If there existed any question as to the accuracy of diagnosis, or if all the clinical facts did not agree, the case was discarded. The results obtained are given in Table 2. The cases are divided according to the classification adopted by the National Association.

If a single plus is used as criterion, a higher percentage of positive results was obtained in the nontuberculous and the more advanced

TABLE 1  
CORRELATION OF AUTOLYSIS AND ANTIGEN-FORMATION

Nitrogen Curve		Antigen Curve							
Days	Mg. N. per c.c.	0.2 c.c.	0.1 c.c.	0.5 c.c.	0.01 c.c.	0.005 c.c.	0.001 c.c.	0.0005 c.c.	0.0001 c.c.
0	0.00	++++	++++	+++	—	—	—	—	—
1	0.00	++++	++++	+++	±	—	—	—	—
2	0.01	++++	++++	++++	+++	±	—	—	—
3	0.02	++++	++++	++++	++++	+++	++	+	—
4	0.03	++++	++++	++++	++++	++++	+++	+	±
6	0.05	++++	++++	++++	++++	++++	++++	++	++
8	0.06	++++	++++	++++	++++	++++	++++	+++	++
10	0.08	++++	++++	++++	++++	++++	++++	+++	+
13	0.15	++++	++++	++++	++++	++++	++++	+++	++

TABLE 2

RESULTS OF COMPLEMENT-FIXATION TESTS ACCORDING TO THE ONE-PLUS (PLAIN TYPE) AND THE DOUBLE-PLUS (BOLD FACE TYPE) CRITERIONS

Cases	Number of Patients Exam- ined	Number of Exami- nations	Negative with Bacillary Emul- sion	Negative with Autol- ysate	Positive with Bacillary Emul- sion	Positive with Autol- ysate	Tubercle Bacilli in Sputum	Results of von Pirquet Tests
Nontuberculous...	25	26	(1 A)* 19 23	19 24	6 2†	7 2†	25—	1-11+
Questioned non- tuberculous.....	11	11	5 6	7 9	6 5	4 2	11—	3-6+
Incipient inactive.	47	50	(2 A) 30 39	35 43	18 9	15 7	46-1+	
Incipient active...	27	30	(5 A) 10 18	14 23	15 7	16 7	19-7+	
Moderately ad- vanced inactive.	12	14	8 10	9 13	6 4	5 1	6-6+	
Moderately ad- vanced active...	47	55	(8 A) 13 24	27 37	34 23	28 18	12-34+	
Far advanced in- active.....	5	5	3 4	3 4	2 1	2 1	3-2+	
Far advanced ac- tive.....	187	216	(28 A) 81 112	(1 A) 110 155	107 76	105 60	15-172+	
Totals.....	361	407	213 280	225 309	194 127	182 98		

\* A = an anticomplementary result.

† One of these cases had received a tuberculin injection for diagnostic purposes a short time previous to drawing the blood.

cases. The double-plus criterion, however, seems to give the more accurate view of the state of affairs, giving as it does a low percentage positive in normals, tho it also lowers the number of positive findings in the clinically certain tuberculosis cases. The results under the double plus may be summed up as follows:

1. Only 1 nontuberculous case out of 25 gave a positive reaction (96% negative by both tests). The second positive had received a tuberculin injection a short time before the test.

2. Of questionable nontuberculous cases 18% were positive in the autolysate test, 45.5% in the emulsion test.

3. Incipient inactive cases gave 14% positive with the autolysate and 18% with the emulsion.

4. Incipient active cases gave the same result with both, 23.3% positive.

5. Moderately advanced inactive cases gave 7.15% positive with the autolysate and 28.6% with the emulsion, while the active cases gave 37.7% with the autolysate and 41.8% with the emulsion.

6. The far-advanced inactive cases gave 20% positive in both tests, while the active cases gave 27.6% with the autolysate and 35% with the emulsion.

A greater percentage of reactions was always obtained in the active cases, but the results seem to indicate, as pointed out by Fraser,<sup>8</sup> that antibodies in free form capable of binding antigen are apparently not always present in the sera of tuberculous individuals, but are most liable to be present in the active form of the disease. (It is significant that all von Pirquet negatives were negative also as regards complement-fixation.) Has complement-fixation, then, any practical value as a diagnostic test for tuberculosis? It can be answered that in conjunction with other findings, complement-fixation makes the diagnosis of tuberculosis definite. It is of value also from a differential diagnostic standpoint in that it points out tuberculosis, when positive, as against syphilis, abscess of the lung, empyema from other causes, carcinoma, bronchiectasis, etc.

Now that the complement-fixation test has been found lacking in point of percentage efficiency as a diagnostic test for tuberculosis, the question arises as to whether or not it is possible by further study to make the test more efficient. With a view to obtaining a higher percentage of positive results a number of autolysates prepared from different strains of virulent tubercle bacilli are being tested on the same sera in order to prove whether or not a polyvalent autolysate antigen

would be more efficient than a monovalent one ; the sera are being drawn at various intervals during the day to see whether there is an especially opportune time for obtaining the antibodies in the sera, as suggested by the periodicity of the temperature curve. Weekly intervals are also being considered. Finally, antigen and antibody tests are being made coincidentally on the same sera, as it has been suggested that possibly in the absence of antibodies a test for antigen may give results.

#### SUMMARY

Virulent cultures of tubercle bacilli free from all foreign substances suspended in sterile salt solution undergo autolysis at incubator temperature as indicated by the liberation of nitrogenous substances, the autolysis reaching a maximum from the 6th to the 8th day.

During the autolysis of virulent cultures of tubercle bacilli there is a coincident liberation of antigenic substances which possess advantages over a suspension of living virulent tubercle bacilli as antigen for complement-fixation tests in tuberculosis.

The examination of 361 persons (25 of them normal, 11 questionably nontuberculous, and 325 definitely tuberculous), using both an emulsion and an autolysate prepared from living virulent human tubercle bacilli as antigens, shows that—

(a) The complement-fixation test for tuberculosis is not absolute, being positive only in about 30% of all the clinically definite cases of tuberculosis both active and inactive. Active cases give a higher percentage of positive results than inactive cases.

(b) The value of the complement-fixation test for tuberculosis lies in the fact that, taken in conjunction with other findings, a definitely positive reaction makes the diagnosis of tuberculosis certain.

(c) It is of value also from a differential diagnostic standpoint in that it indicates tuberculosis, when positive, as against syphilis, carcinoma, abscess of the lung, empyema from other causes, bronchiectasis, etc.

The practical absence of a reaction in nontuberculous cases makes this test, when positive, of far greater value in the diagnosis of tuberculosis than any of the biologic tests for tuberculosis thus far discovered. A positive test was never obtained in the absence of a positive von Pirquet reaction, but a large percentage of clinically normal individuals giving positive von Pirquet reactions were negative in fixation tests.

# STUDIES ON ACQUIRED TYPHOID IMMUNITY\*

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The experiments reported in this paper were undertaken primarily to determine whether or not immunity following infection or inoculation is due to a training or an exercise of the cells in the production of antibodies so that a later invasion will cause a more rapid or a more prolific formation of immune bodies, with a consequent destruction of the invading organism before it can gain a foothold.

This work, which we started 6 years ago, is not yet completed, but it is far enough advanced to warrant a statement of the results obtained. Our studies thus far have been limited to the effects of inoculation with typhoid vaccine and later re-inoculation with either dead or living typhoid bacilli. The entire subject falls into 2 parts: (1) An investigation of the changes produced in the blood serum by immunization, as determined by a study of the amounts of agglutinin, bactericidin, and opsonin in the blood serum of rabbits before, and at regular intervals after, inoculation. (2) An investigation of the effects of re-inoculation as determined by (a) a comparison of the amounts of agglutinin, bactericidin, and opsonin present in the blood serum of rabbits before, and at regular intervals after, the injection of antityphoid vaccine, with the amounts produced after re-inoculation of the animals with similar doses of the identical vaccine after the immune bodies have disappeared from the blood; and, (b) a comparison of the amounts of agglutinin, bactericidin, and opsonin found in the blood serum of rabbits receiving sublethal doses of virulent typhoid bacilli with the amounts found in the blood of previously immunized rabbits after they have received similar sublethal doses.

## HISTORICAL REVIEW

Wollstein<sup>1</sup> studied the duration of the immune bodies present in the blood after typhoid inoculation, and found that in a series of 24 inoculated persons the bactericidin reached its greatest height 1 month after the 3rd inoculation, to fall rapidly during the next 2 months. Of 19 cases studied longer, 8 were negative for bactericidin 10 months after inoculation, and 15 after 13 months. The agglutinin disappeared even more quickly, for after 4 months only 2

\* Received for publication March 3, 1916.

<sup>1</sup> Jour. Exper. Med., 1912, 16, p. 315.

agglutinated the typhoid bacillus in a dilution of 1:160, and no agglutinin was present 13 months after vaccination.

Cole<sup>2</sup> found that a guinea-pig which had been injected with typhoid bacilli formed antibodies more rapidly on being re-injected, while a control animal responded slowly, with a less abundant formation of antibodies.

Moon<sup>3</sup> studied the agglutinin in human subjects who had been previously inoculated with antityphoid vaccine or who had suffered previously from an attack of typhoid fever. To quote from his article: "As shown by these curves there is a distinct difference in the rapidity with which human beings who have previously been immunized form antibodies as compared with those who have not. It would seem as if the previously immunized person remains more sensitive to the antigenic influence of typhoid bacilli and responds more quickly by the production of antibodies when the bacilli are introduced into the system. Such a result is in keeping with our knowledge of the phenomena of allergy. That this condition would contribute to the resistance against typhoid and the readiness with which the body would overcome the bacilli is easily understood."

#### TECHNIC

*Agglutination.*—The agglutination tests were made by the microscopic method. A 24-hour peptone-solution culture of a very sensitive strain of a typhoid bacillus that had been in the laboratory for several years was used. This organism was one of those present in the polyvalent vaccine employed in all the experiments. The serum, unless otherwise stated, had been heated beforehand for one-half hour at 56 C. Only those dilutions were considered positive which caused complete clumping with cessation of all motility within 2 hours. All tests were controlled by a hanging drop of the peptone culture.

*Bactericidin.*—The bactericidin was determined in vitro by a slight modification of the method first described by M. Neisser.<sup>4</sup> In each of a series of small test tubes was placed 0.25 c.c. of a dilution (a different dilution in each case) of inactivated serum from the animal that was to receive, or had received, the inoculations of antigen. To each tube was added 0.5 c.c. of a 1:10,000 dilution of a 24-hour broth culture of the typhoid bacillus that was employed in the agglutination tests. The dilution of the serum and that of the typhoid culture were always made by the addition of 0.85% sterile salt solution. A dilution of complement was prepared by mixing 1 c.c. of fresh rabbit serum, 6.2 c.c. of salt solution, and 4.8 c.c. of sterile broth. To each tube 0.25 c.c. of this diluted complement was added, making the total quantity 1 c.c. The tubes were thoroughly shaken and incubated at 37 C. for 3 hours. They were again thoroughly shaken and the contents of each tube poured into a Petri dish and mixed with 10 c.c. of melted neutral agar that had been cooled to 40 C.

For each series of tubes 3 controls were made. First, a typhoid control, containing 0.5 c.c. of the diluted typhoid culture, 0.1 c.c. of sterile broth, and 0.4 c.c. of salt solution. This immediately after being made was poured into a Petri dish and mixed with neutral agar. It represented the number of typhoid bacilli planted in each tube. Second, a 3-hour control was made in the same manner as the typhoid control, but incubated with the tubes containing the serum to show the increase in the number of bacteria that occurred during the 3 hours. The 0.1 c.c. of broth added to each of these controls

<sup>2</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1904, 66, p. 367.

<sup>3</sup> Jour. Infect. Dis., 1914, 14, p. 56.

<sup>4</sup> Ehrlich: Studies on Immunity, 1906, p. 348.

equalled the amount in the diluted complement of the other tubes. Neisser<sup>4</sup> lays stress on the presence of the broth in these experiments, claiming that "it suffices to balance disturbing variations of the osmotic pressure." Third, a complement control was set up, consisting of 0.25 c.c. of diluted complement, 0.5 c.c. of the diluted typhoid culture, and 0.25 c.c. of salt solution. This was incubated with the other tubes and usually showed fewer colonies than the 3-hour control. The difference in the number of colonies represented the inhibitory action of the complement-bearing serum.

All plates were incubated for 48 hours, at the end of which the colonies on each were counted. Where the number was large we counted them with the low-power lens; in such cases the number of colonies in each of several different fields was determined, these numbers averaged, and the result multiplied by the number of fields on the plate.

This bactericidal test, as pointed out by Neisser, is subject to wide variations, and under no circumstances can it be considered very sensitive or accurate. A decrease in the number of colonies may be due to agglutination of the organisms. It is therefore necessary to make several dilutions of the amboceptor, and to regard as bactericidal only those that cause a marked diminution of colonies while higher or lower dilutions show large numbers of bacteria. The more concentrated solutions of amboceptor often fail to produce any bactericidal effect owing to an excess of the intermediary body and a consequent deflection of complement (Neisser-Wechberg phenomenon).

While Neisser estimated the bactericidal effect by dividing his plates into those containing no colonies, those containing 100, and those containing 1,000 and innumerable colonies, we have adopted the following arbitrary standard of what we believe may be considered bactericidal action: Since, other things being equal, the difference between the number of colonies on the 3-hour control and that on the complement control was due to the inhibiting effect of the normal rabbit serum, we determined in each instance the percentage of this reduction, deducted a proportionate number from the "typhoid control," and then regarded as bactericidal only those dilutions that gave plates containing not more than one-fiftieth the number of colonies on the corrected typhoid control. For example, if the 3-hour control plate contained 100,000 colonies, the complement control plate 75,000, and the typhoid control plate 20,000, then the normal serum inhibited 25,000, or 25% of the total number of bacteria in the 3-hour control. As the typhoid control contained 20,000 bacteria, each tube in the series was seeded with this number of organisms; but if 25% were restrained by the action of the complement-bearing serum there remained only 15,000 organisms to be acted on by each dilution of antityphoid amboceptor. Hence only the tubes that contained 300 colonies or less would be considered to show the effects of typhoid bactericidin.

We believe that this gives a generous margin for all the factors that might influence results. Altho agglutination may cause some error, the charts demonstrate that many of the sera were bactericidal at much higher dilutions than those at which they were agglutinative. The dilutions of bactericidin given in the tables are those of the amboceptor before it was placed in the tubes; as each of these contained 0.25 c.c. of this serum together with 0.5 c.c. of the culture and 0.25 c.c. of complement, the actual dilution of each immune serum was 4 times greater than that given in the charts.

*Opsonin.*—In our opsonic estimations we again used an arbitrary standard. The dilution method, modified after Neufeld, was employed as follows: An emulsion of the same strain of the typhoid bacillus as that used in the

agglutination and bactericidal tests was made by washing off with normal salt solution the 24-hour growth on an agar slant. Equal quantities of this emulsion, of leukocytic cream, and of one of the various dilutions of serum were mixed together in capillary tubes, which, after sealing, were incubated for 20 minutes. The ends were then broken and smears made and stained with Jenner's stain. The dilutions that caused 50% or more of the leukocytes to be phagocytic were regarded as being opsonic. The dilution as expressed in the text and the charts is that of the serum before the addition of bacterial emulsion and leukocytic cream; the actual dilution is therefore 3 times as great. Our experience with the opsonic tests was far from satisfactory; phagocytosis occurred only at dilutions much lower than those causing agglutination and this naturally would lead to erroneous results as the fields became obscured by the agglutinated bacilli.

The charts show the highest dilution at which each of the immune substances was active on the various days on which the blood was collected. The figures in the left-hand margin of each of these charts correspond to a position half-way between the lines.

## I

In the study of the amount of agglutinin, bactericidin, and opsonin in the blood serum of rabbits before, and at regular intervals after, the inoculation with antityphoid vaccine, 11 rabbits were inoculated with 3 or 4 doses of typhoid vaccine. The size of the dose and the intervals between injections varied, but in most instances from 7 to 10 days elapsed between the successive inoculations.

A polyvalent vaccine made with 10 different strains of *B. typhosus* was used in all the experiments. The injections were made subcutaneously in the region of the groin after the hair had been shaved off, and the area washed with a solution of mercuric chlorid followed by sterile water.

Before collection of the blood, the ear was shaved and scrubbed with soap and water, sterilized with bichlorid of mercury and sterile water, and dried with sterile gauze. One of the more prominent blood vessels of the ear was punctured by a small blood stylet and the blood collected by means of a Wright capillary pipet. Bleeding was facilitated by placing under the abdomen of the rabbit a rubber bag filled with warm water. As soon as collected the blood was centrifugated and the serum inactivated by heating at 55 C. for one-half hour. If the blood was not used immediately, it was kept at a temperature of 10 C. until needed.

The 3 following experiments illustrate this section of the work:

*Rabbit B.*—This rabbit was first inoculated with 100,000,000 dead typhoid bacilli, 8 days later with 200,000,000, and again after 8 days with 300,000,000 dead bacteria. Blood was collected before inoculation and daily afterward; also on the 1st, 3rd, 5th, and 7th days after the second and third inoculations; 2 weeks and 1 month after the last inoculation, and at monthly intervals thereafter. The serum before inoculation agglutinated at a dilution of 1:4, but was not bactericidal. The curve for the antibodies following inoculation is shown in Chart 1.

*Rabbit D.*—This rabbit was inoculated with doses of 200,000,000, 400,000,000 and 600,000,000 dead typhoid bacilli, respectively, at intervals of 1 week. Blood

was collected before the first inoculation and every day for a week succeeding; on the 1st, 3rd, 5th, and 7th days after the second; and on the 2nd, 3rd, and 6th days after the third; also at the end of 2 weeks and after the 1st, 2nd, 3rd, 4th, 5th, 8th, and 9th months following the last injection. Before immunization the serum agglutinated at a dilution of 1:10, was not bactericidal, but was opsonic when undiluted. The result of the inoculations is shown in Chart 2.

*Rabbit H.*—The animal was injected at weekly intervals with doses of 250,000,000, 500,000,000, and 750,000,000 dead typhoid bacilli, respectively. Blood was collected before the first inoculation and daily following; on the 1st, 3rd, 5th, and 7th days after both the second and third inoculations; and also 2 weeks after the last, and then at monthly intervals. Before immunization the serum agglutinated at a dilution of 1:4 and was both bactericidal and opsonic when undiluted. Chart 3 shows the result of the experiment.

The 11 experiments with rabbits showed that the antibodies produced after the injection of antityphoid vaccine disappeared from the blood in from 6 to 340 days; on the other hand, figures indicate that the immunity conferred by inoculation of human beings usually lasts several years.

## II

The following experiments were made to determine whether or not the injection of dead typhoid bacilli causes a sensitization of the cells concerned in the production of the immune bodies. As before stated, this problem was approached by a comparison of the amounts of agglutinin, bactericidin, and opsonin in rabbit serum before, and at regular intervals after, inoculation with antityphoid vaccine, with the amounts formed after re-inoculation with similar doses of the same vaccine after the antibodies had disappeared from the blood. Six rabbits were re-inoculated with similar doses of the same vaccine with which they had originally been immunized. The comparison between the antibody-formation after inoculation and that after re-inoculation is shown in Charts 1 and 3.

A second approach to the subject was made in a comparison of the amounts of agglutinin, bactericidin, and opsonin appearing in serum of rabbits receiving sublethal doses of virulent typhoid bacilli with the amounts appearing in the blood of previously immunized rabbits receiving similar sublethal doses.

Three rabbits were inoculated with a sublethal dose of a virulent culture of *B. typhosus* and one previously vaccinated rabbit was inoculated with an equal amount of the same typhoid culture. Rabbit D was selected to control the inoculations of sublethal doses of living typhoid cultures because, as measured by antibody-production, it had

CHART I  
RABBIT B.  
Reinoculation.

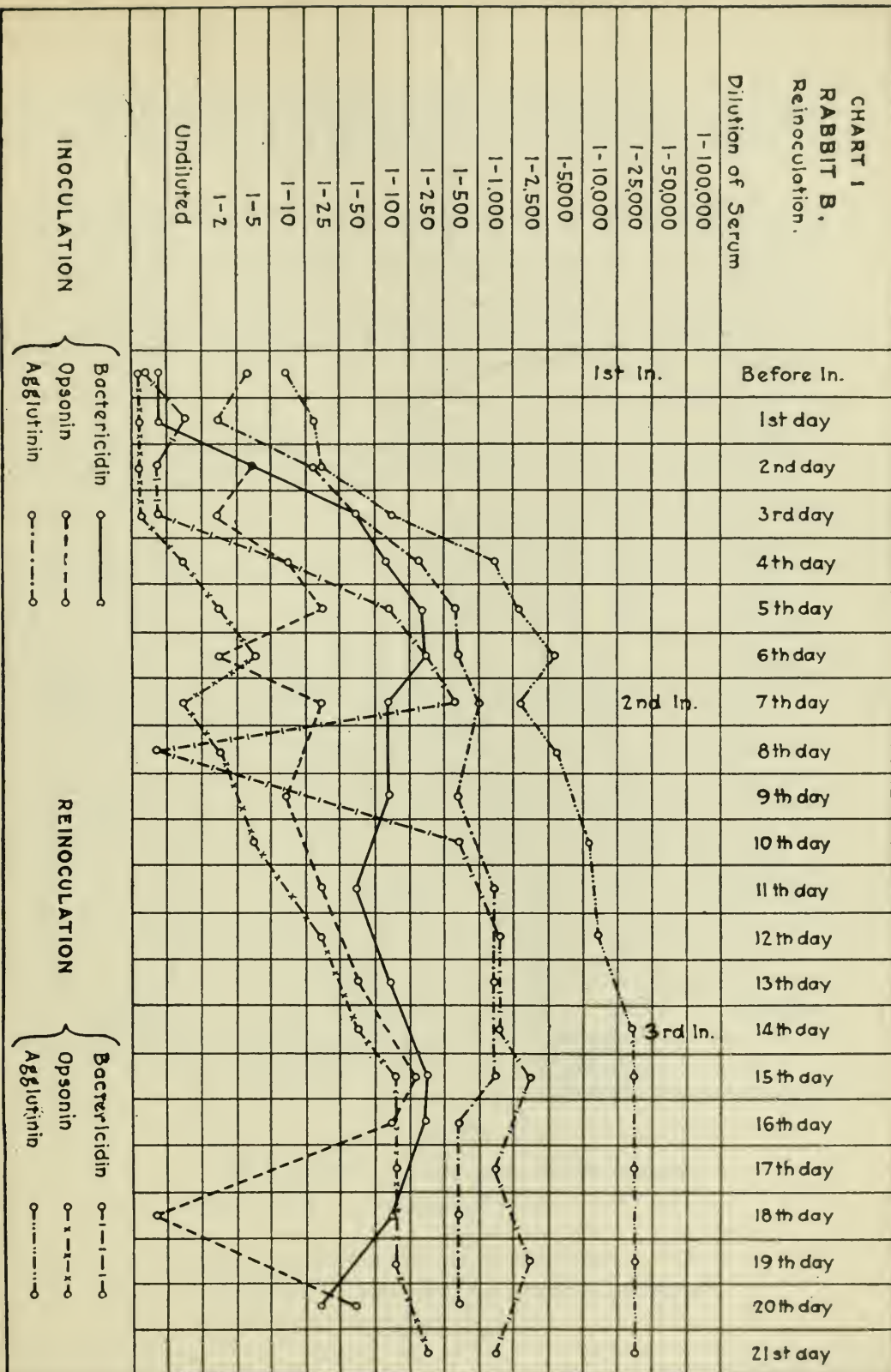


CHART 2  
RABBIT D.  
Reinoculation.

Dilution of Serum

Before In.

1st day

2nd day

3rd day

4th day

5th day

6th day

7th day

8th day

9th day

10th day

11th day

12th day

13th day

14th day

15th day

1-25,000

1-10,000

1-5,000

1-2,500

1-1,000

1-500

1-250

1-100

1-50

1-25

1-10

1-5

1-2

Undiluted

INOCULATION

REINOCULATION

Bactericidin

Opsonin

Agglutinin

Bactericidin

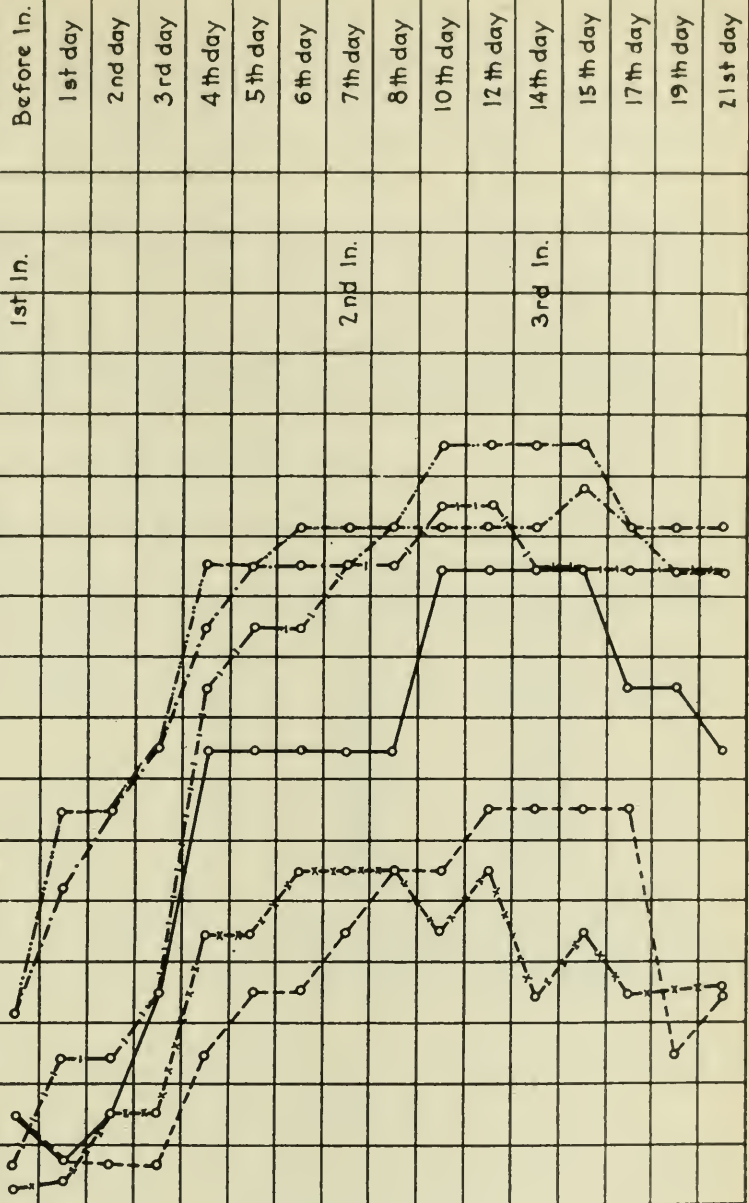
Opsonin

Agglutinin



**CHART 3**  
**RABBIT H.**  
**Reinoculation.**

Dilution of Serum



INOCULATION

Bactericidin —○—○—  
Opsonin —○---○—  
Agglutinin —○-.-.-○—

REINOCULATION

Bactericidin —○-|-|-|-○—  
Opsonin —○x-x-x-x○—  
Agglutinin —○-.-.-○—

TABLE 1

TIME ELAPSING BEFORE THE MAXIMAL FACTOR WAS ATTAINED BY EACH OF THE ANTIBODIES FOLLOWING THE PRIMARY AND SECONDARY SERIES OF INOCULATIONS

Rabbit	Days After Inoculation			Days After Re-inoculation		
	Agglutinin	Bactericidin	Opsonin	Agglutinin	Bactericidin	Opsonin
A	27	27	52*	16	26	22
B	11	5	15	14	15	21
C	16	7	10	18	16	
D†	14	8	14	4	5	7
F	21	21	21			
G	11	8	15			
H	15	10	12	10	10	6
J	15	21	7			
K	27	27	7			
L	8	6	6	6	8	19
M	14	14	6	6	7	7

\* No opsonic determinations were made from the 13th to the 52nd day.

† This animal was re-inoculated with sublethal doses of living typhoid bacilli.

TABLE 2

TITER OF MAXIMAL FACTOR FOR EACH OF THE ANTIBODIES AFTER THE PRIMARY AND SECONDARY SERIES OF INOCULATIONS

Rabbit	First Series of Inoculations			Second Series of Inoculations		
	Agglutinin	Bactericidin*	Opsonin*	Agglutinin	Bactericidin*	Opsonin*
A	1:1000	1:1000	1:25	1:2000	1:1000	1:100
B	1:1000	1:250	1:100	1:25000	1:2500	1:250
C	1:3000	1:250	1:50	1:10000	1:1000	
D†	1:500	1:100	1:25	1:2000	1:2500	1:100
F	1:3000	1:50000	1:500			
G	1:3000	1:10000	1:1000			
H	1:3000	1:1000	1:50	1:5000	1:2500	1:25
J	1:3000	1:5000	1:25			
K	1:4000	1:2500	1:100			
L	1:2000	1:500	1:10	1:1000	1:2500	1:50
M	1:50000	1:50000	1:50	1:100000	1:100000	1:2500

\* The dilution of the bactericidin and of the opsonin is expressed in terms of that of the serum added to the other substances: the final dilution in each case would be 4 times and 3 times these figures, respectively.

† This animal was re-inoculated with sublethal doses of living typhoid bacilli.

TABLE 3

TIME ELAPSING BEFORE MAXIMAL FACTORS WERE ATTAINED FOLLOWING INOCULATION AND TIME ELAPSING BEFORE THE SERUM REACHED THE SAME ACTIVITY, AS GUAGED BY DILUTION, AFTER RE-INOCULATION

Rabbit	Days After Inoculation			Days After Re-inoculation		
	Agglutinin	Bactericidin	Opsonin	Agglutinin	Bactericidin	Opsonin
A	27	27	52*	5	26	18
B	11	5	15	4	6	Between 10 and 21
C	16	7	10	Between 10 and 12	12	
H	15	10	12	Between 8 and 10	7	†
L	8	6	6	†	5	5
M	14	14	6	Between 5 and 6	5	Between 3 and 4

\* No opsonic determinations were made from the 15th to the 52nd day.

† The opsonin after re-inoculation did not, during the course of the experiment, reach the same maximal factor.

‡ The agglutinin after re-inoculation did not, during the course of the experiment, reach the same maximal factor.

developed a very high grade of immunity following the primary inoculations, and it had been immunized longer than any of the other animals.

The following experiment shows the result of the injection of a sublethal dose into an unvaccinated rabbit, while Chart 2 clearly shows the result for the vaccinated animal.

*Rabbit W.*—Inoculated subcutaneously with 5 c.c. of a 24-hour broth culture of living typhoid bacilli. Blood was collected before the inoculation, and on the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 9th, 11th, 13th, and 15th days thereafter. The serum did not cause agglutination before inoculation nor until the 4th day following, when clumping occurred at a dilution of 1:20. From the 6th until the 9th day the agglutinin increased steadily, being active at a dilution of 1:1,000 on the 9th day; from this time until the termination of the experiment there was no further rise in the curve.

No bactericidin was demonstrable in the serum of Rabbit W before inoculation. Seven days later the bactericidin was active at a dilution of 1:2 and from this time it increased steadily until the 13th day, when it was active at a dilution of 1:250; no difference was noticed on the 15th day, the last day on which blood was drawn.

No opsonin was found in the serum until the 11th day after inoculation, when phagocytosis occurred at a dilution of 1:5; on the 13th and 15th days the opsonin was active at a dilution of 1:10.

#### SUMMARY AND CONCLUSION

Of the 15 rabbits used in this work, all save 1 showed the presence of normal agglutinin in their sera; 7 of the sera were agglutinative at a dilution of 1:4, 4 at 1:10, and 1 at 1:20. Few of the animals, however, had normal bactericidin or opsonin in their sera. In no instance was bactericidin demonstrable in a serum diluted higher than 1:5, while opsonin could be demonstrated only when undiluted serum was used.

The agglutinin was usually the first of the immune bodies to increase following inoculation and it generally persisted for a longer time than either of the other two. The bactericidin, as a rule, was the second to increase and was usually present for a longer time than the opsonin.

With 2 exceptions the agglutinin attained its highest activity, as gauged by dilution, several days earlier after re-inoculation than after inoculation, and, with 1 exception, it reached much earlier the degree of activity corresponding to the maximal dilution factor after inoculation. In illustration of these statements Tables 1 and 3 are worthy of attention.

While it is evident from an analysis of the results in the cases of Rabbits A, B, C, H, L, and M—the animals re-inoculated with typhoid vaccine—that the bactericidin reached its maximal factors on the average a few days earlier following the primary series of inoculations, it must be remembered that the maximal factors attained after the 2nd series were almost invariably greater, as is clearly to be seen in the tables.

Again, the average maximal factor for each of the immune bodies was greater following re-inoculation than following the primary injections; in the case of the agglutinin, 1:23000 as compared with 1:10000; of the bactericidin, 1:18000 as compared with 1:9000; and of the opsonin, 1:585 as compared with 1:65.

Different grades of immunity, as measured by the antibodies considered, were produced in the different animals irrespective of their size, of the dose of vaccine employed, and of the intervals between inoculations.

There was no definite relation between high maximal factors of the antibodies and the length of time after inoculation that these bodies could be demonstrated in the serum, since in some instances the immune substances disappeared much earlier from the serum of animals which reacted at high dilutions than they did from the serum of those which reacted at comparatively low dilutions.

The experiments were too few to form the basis of any dogmatic statement, but it would seem that the immunity following inoculation is due to a training of the cells in the production of antibodies so that afterward they yield these more prolifically.

# THE CAUSATION OF GASTRIC AND DUODENAL ULCER BY STREPTOCOCCI \*

PLATES 5 TO 14

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Lesions of the stomach have been produced experimentally by excision; by tying a fold of mucous membrane with a string; by local application of the cautery, and of corrosive chemicals, such as silver nitrate and nitric acid;<sup>1</sup> by the introduction of very hot gruels;<sup>2</sup> by the submucous injection of silver nitrate,<sup>3</sup> nitric acid, adrenalin, alcohol,<sup>4</sup> and gastro-toxic serum,<sup>5</sup> and by the injection of lead chromate<sup>6</sup> and fat<sup>7</sup> into the gastric artery; but since these lesions heal promptly in from 5 to 21 days, according to their severity,<sup>1,2,6</sup> and since the methods used are so foreign to what could occur, valuable tho the results are in one respect or another, they have little bearing on the problem of ulcer of the stomach as it occurs in man.

The hemorrhages, erosions, and ulcerations observed during severe intoxication following systemic injections of snake venom, pilocarpin, atropin, chloroform, phenol, copper sulfate,<sup>8</sup> bile, and bile salts,<sup>9,10</sup> B-tetra-hydronaphthylamine,<sup>11</sup> adrenalin,<sup>12</sup> diphtheria toxin,<sup>13,14</sup> and culture filtrates of various bacteria, while having some parallelism in acute ulcer during severe or fatal intoxications or infections, have little bearing on the problem of the usual ulcer of the stomach in man.

The same may be said of the lesions of the stomach which develop commonly in animals during the moribund condition following adrenalectomy.<sup>15,16,17,18,19,20</sup>

Ulcer of the stomach has been produced by interference with its nerve supply (hence the blood supply), by section either of the vagus or of the sympathetic nerves, or of both, and by section of the spinal cord.<sup>21,22,23,24</sup> Most observers, however, have obtained only doubtful results in this field. The studies of Vedova<sup>22</sup> and Durante<sup>23</sup> merit special mention. They have shown that in dogs and rabbits ligation or section of the sympathetic nerves is followed somewhat regularly by acute ulcer of the stomach. In some instances they have observed ulcers which were chronic in type, but not in time, the animals surviv-

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ing the operation for a short time only. The lesions in the stomach were due according to Durante to spasm of the blood vessels as the result of an over-production of adrenalin, the adrenals showing marked congestion and hemorrhage following ligation of the splanchnic nerves. But since there is no evidence of increased adrenal function, as manifested especially by an increased blood pressure, in patients with ulcer, and since the death of the animals occurred as a result of the method employed, it is difficult to see how these results, suggestive as they are, can be applied, for in ulcer the interference with the nerve supply of the stomach is necessarily slight.

The infectious origin of ulcer, while not generally accepted, has had adherents for many years. Experimentally, ulcer has been produced by intravenous injections of the pyocyaneus bacillus,<sup>25</sup> the dysentery bacillus,<sup>26</sup> the lactic-acid bacillus,<sup>27</sup> and the colon bacillus.<sup>28, 29, 30</sup>

Ulcer was found to occur as a part of a general pyemia following intravenous injections (1) of pus by Lebert<sup>31</sup> (1857) and by Cohn<sup>32</sup> (1860), (2) of streptococci and staphylococci by Letulle,<sup>33</sup> and (3) of pneumococci by Bezançon and Griffon.<sup>34</sup> It is a well-known fact that ulcer of the stomach in man occurs not infrequently during severe or fatal infections of various kinds, particularly streptococcal infections.<sup>35, 36, 5</sup> Bacteria have been repeatedly demonstrated in the edges and floors of ulcers—Boetcher<sup>37</sup> in 1874 being the first to do this—but these have usually been considered secondary invaders. Dudgeon and Sargent<sup>38</sup> isolated a diplostreptococcus from the edges of the ulcers and from the peritoneal exudate in 4 of 9 cases of peritonitis following perforation. The peritonitis was of a mild grade corresponding to the low virulence of the strains isolated. Hutyra and Marek<sup>39</sup> state that ulcer of the stomach is found in domestic animals dead from "catarrhal fever," and in purpura hemorrhagica. Bolton<sup>5</sup> states that probably the commonest cause of necrosis of the mucous membrane and resulting acute ulcer of the stomach is bacterial infection, that the infection occurs through the blood stream, and that the necrosis is due to the direct effect upon the tissues of the bacterial poison, alone or together with the gastric juice. Letulle reports a case of ulcer following chronic abscess of the maxillary antrum, and notes the occurrence of acute ulcer in other cases in which there were local septic foci. This raises the important question, to quote Bolton, "whether many cases of simple acute ulcers owe their origin to some local septic focus which is so commonly unrecognized or unheeded." Recently numerous

observers have emphasized the etiologic relation of focal infection to ulcer of the stomach.

In 1913, I reported<sup>40</sup> some experiments which showed that streptococci, quite irrespective of their original source, when of a certain grade of virulence, exhibit affinity for the gastric mucous membrane, producing a localized infection and ulcer. The report at that time was summarized as follows:

Intravenous injection of streptococci of the proper grade of virulence may be followed by ulcer of the stomach and duodenum. The ulceration is due to a localized infection and secondary digestion. The ulcers are usually single and deep with marked tendency to hemorrhage and perforation, and in many respects resemble gastric ulcer in man. When we take into consideration this close resemblance, that injection of streptococci which have grown in tonsils produce the lesions, and that the virulence of the germs when the affinity for the stomach is greatest, is of such a character that a general infection does not occur, it appears altogether reasonable to suppose that in man gastric ulcer may be caused by streptococci also. The supposed relation between infected tonsils or gums and gastric ulcer may be due not to the swallowing of bacteria, as usually supposed, but to the entrance into the blood of streptococci of the proper kind of virulence to produce a local infection in the wall of the stomach. Many other observations might be cited, such as associated infections of the gall bladder and appendix, which suggest that gastric ulcer may be due to streptococci.

Before it could be accepted, however, that the usual ulcer of the stomach in man is due commonly to a local hematogenous streptococcal infection, it was necessary to show, first, that in this type of ulcer, these organisms are commonly present to the exclusion of other bacteria; and second, that the streptococci isolated from the ulcer wall, as well as those from foci of infection in patients having ulcer, produce, when injected into animals under otherwise normal conditions, ulcers of the stomach and of the duodenum resembling those in man.

By the use of a special technic the first requirement has been covered, and reported.<sup>41</sup> Streptococci have been demonstrated in the tissue or isolated in cultures (often in pure form) in 42 of 54 typical chronic ulcers in man. The second requirement, as I have pointed out recently,<sup>42</sup> has also been fulfilled. I wish now to give in greater detail the results of my experiments.

#### TECHNIC

The technic of making cultures from ulcer is described in another paper.<sup>41</sup> In collecting material for cultures, great effort was made to obtain bacteria from the depths of the foci of infection with as little surface contamination as possible. This is of great importance. The bacteria for injection were grown in tall columns (12 cm.) of ascites dextrose broth at 35-37 C., for from

18 to 24 hours, centrifugated, the clear broth poured off, and the bacteria suspended in salt solution so that 1 c.c. of the suspension contained the growth from 15 c.c. of the broth culture. Blood-agar-plate cultures and smears were made of the material obtained directly from the ulcer or the focus, from the broth cultures, and from the suspensions immediately before injection, to determine the viability and identity of the organisms present.

In some instances, small doses of the broth culture and cultures from blood-agar slants were injected. Bacteria from the ulcers, when injected into animals, were usually in the second culture, from single colonies (from 2 to 78 days old) in the original shake cultures. In the case of strains from foci of infection, the bacteria used for injection were usually from the primary culture, from 18 to 48 hours after the inoculation of material from the focus directly into tall columns of ascites dextrose broth. The broth culture filtrates were injected in some instances to determine the presence or absence of an ultramicroscopic virus. They showed only slightly greater tendency to produce lesions in the stomach than did filtrates from other streptococcus cultures.

Routine cultures were made in the case of the animals from the blood (0.5 c.c.), the joint fluid (usually from 2 or more joints), the bile and from emulsions of the ulcers, of the areas of hemorrhage, and often from the adjoining normal mucous membrane. Cultures from various other tissues and from the blood at intervals during life, were made in selected instances. The technic for tissue cultures from animals was similar to that employed in making cultures from ulcers from patients.

No special attention was paid to the diet of the animals. The injections were made usually in the latter part of the day, some hours after the feeding period. Except for feeding experiments, and the local and intraperitoneal injections of the streptococci, the bacteria were injected intravenously, the injections being made rather rapidly through a fair-sized needle (23 gage). It was the rule to inject at least 2 animals with a given strain, one receiving a small dose, the other a larger dose. Because of the low virulence of the strains, the dose was relatively large, consisting in the main (for rabbits and dogs) of the growth from 5 to 25 c.c. of the broth culture per kilo of weight. In special instances much smaller doses sufficed to produce ulcer.

For sections the tissues from the ulcers in man and from experimental ulcers were fixed in 10% formalin or in Zenker's fluid, imbedded in paraffin, and stained chiefly with hematoxylin and eosin, methylene blue and eosin, or with neutral gentian. Gram-Weigert's method was used as a routine for the staining of bacteria, care being exercised not to decolorize the sections further than to a pale blue.

## REVIEW OF CASES AND EXPERIMENTS

### CASE 773

A man, 53 years of age, with typical symptoms of duodenal ulcer of 3 years' duration was operated on by Dr. Bevan Oct. 27, 1913. There was excised a hard, indurated, much thickened and adherent duodenal ulcer, which was perforating against the gall-bladder. Cultures were made at once. Smears from the emulsion showed a few gram-staining cocci and diplococci. The cultures yielded fully 1,500 colonies of staphylococci and approximately 20 colonies of a short-chained nonhemolyzing nonadherent streptococcus.

The streptococcus (Fig. 1) as isolated, was injected into 2 dogs and 2 rabbits. The dog receiving the large dose died in 48 hours, showing marked

distention of the stomach with gas containing a large amount of carbon dioxide, and hemorrhages of the mucous membrane of the stomach with ulceration in two areas. The other dog, 13 weeks later, died of distemper. It showed one round deep sharply circumscribed, somewhat indurated ulcer of the duodenum, 5 mm. in diameter, 2 cm. beyond the pyloric ring (Fig. 7). The floor of the ulcer in the center consisted only of the thickened and adherent peritoneal coat. No other lesions could be made out. One rabbit developed one ulcer in the pylorus, together with myositis and beginning endocarditis. The other did not show lesions of the stomach, but showed a focal nephritis and myocarditis.

The staphylococci failed to produce lesions of the stomach, but produced a focal nephritis, and, in one rabbit, cholecystitis.

The feeding of a large amount of cultures of these strains mixed with chopped meat and particles of dried splintered bone, failed to produce lesions in the stomach or duodenum in the 3 dogs so treated.

After 1 animal passage, the streptococcus produced acute ulceration of the stomach in a rabbit, and a chronic ulcer causing death from hemorrhage in the only dog injected (see report for Dog 41).

After 2 animal passages, the streptococcus was injected into 2 dogs. One developed 3 hemorrhagic ulcers—2 in the duodenum and 1 in the pyloric end of the stomach—cholecystitis, pancreatitis with fat necrosis, enteritis, and myocarditis. The other dog developed no lesions of the stomach or duodenum, but showed fatty degeneration of the liver and ulcerative colitis.

After 3 animal passages, the streptococcus produced 2 ulcers of the stomach in one of 2 dogs injected, together with enteritis and colitis, and intussusception of the ileum into the cecum. Cultures from the blood of these dogs yielded both nonhemolyzing and hemolyzing streptococci. The nonhemolyzing strain in the next passage produced no lesions of the stomach in the 3 rabbits injected, one rabbit developing arthritis, and another cholecystitis; the hemolyzing strain likewise produced no lesions of the stomach, and caused a suppurative arthritis in 2 rabbits and 1 dog. The dog showed what appeared to be a phlegmonous gastritis, but no ulceration.

After 5 animal passages, the nonhemolyzing strain was injected into 3 guinea-pigs, 2 dogs, and 1 rabbit. All but 2 of these animals showed hemorrhages in the lungs. The rabbit and the dogs showed hemorrhages of the gall-bladder and mild arthritis. In 1 dog there was marked degeneration of the liver. Cultures from the joint fluid in these were negative. Cultures from the livers which showed no gross changes, remained sterile, while those from the liver in which there was degeneration, yielded a large number of streptococci.

*Dog 41.*—Injected Nov. 11, 1914, with the growth from 150 c.c. of a dextrose-broth culture of the streptococcus after one animal passage. Cultures from the blood the day after injection were negative.

Nov. 25.—Dog seemed well, but had lost in weight, and was less active than usual.

Jan. 3.—Died at 9:00 a. m., 53 days after a single injection. Emaciation: tissues everywhere pale, hemoglobin 30%; blood remained unclotted for 30 minutes. There were one small round deep ulcer with elevated and indurated margins and hemorrhagic base, which extended through the mucous membrane; one healing ulcer in the stomach and one in the duodenum; and one active infected ulcer 1 cm. beyond the pyloric ring (2 by 5 mm.). The margins of the latter were infiltrated and opaque, and the peritoneum opposite showed

thickening and adhesions. The liver disclosed fatty infiltration and localized fibrous myocarditis. The other organs revealed no changes. A Weber test for occult blood in the dark brown contents of the lower bowel was strongly positive.

Cultures from blood, joint fluid, and kidney remained sterile, while those from bile yielded 15 colonies of nonhemolyzing streptococci.

*Dog 37.*—Injected intravenously Nov. 3, 1913, with the growth from 280 c.c. of a dextrose-broth culture of the streptococcus in its second culture.

Nov. 7.—Cultures from blood gave the streptococcus injected.

Nov. 16.—Growth from 120 c.c. injected intravenously.

Nov. 19.—Culture from blood made the day after injection yielded streptococci.

Nov. 28.—The dog had grown thin, did not eat with a relish, but otherwise seemed fairly well.

Feb. 13.—Found dead from distemper. There was an acute pneumonia with pleuritis. The stomach was normal—no scars indicating healed ulcers could be made out. In the duodenum, 2 cm. from the pyloric ring, there was a round deep sharply circumscribed ulcer, 5 mm. in diameter (Fig. 7). The margins were undermined and adherent to the muscular layer. The floor in the center consisted of the thickened and adherent peritoneal coat.

Sections showed marked invasion of the muscular coat by connective tissue, and thickening of the peritoneal coat (Fig. 8, a and b). The mucous membrane at the margin of the ulcer revealed round-cell infiltration in areas at the junction of the uninvolved portion. The mucous glands evinced little change. No thrombosed blood vessels could be made out. A search for bacteria revealed a few diplococci (Fig. 9).

#### CASE 779

Chronic ulcer of the stomach in a woman 43 years of age. The symptoms of ulcer had been present for 12 years. She had had the usual periodic attacks of exacerbation and remissions, and was finally operated on by Dr. Ochsner Oct. 29, 1913. There was an indurated crater-like ulcer, 3 cm. by 1 cm., at the pyloric end of the stomach. The pylorus and the lymph gland draining the area were removed and cultured. The margin and base of the ulcer were indurated, the base clean and smooth. The mucous membrane was adherent and not undermined. The ulcer appeared to be healing.

Smears from the base of the ulcer showed leukocytes, epithelial cells, yeast cells, and gram-positive cocci, mostly within leukocytes.

Oct. 30.—Cultures from emulsion of the ulcer, made after the surface was sterilized, yielded approximately 180 colonies of small cocci, which appeared singly and in masses, and approximately 50 colonies of a short-chain streptococcus, while those from the lymph gland gave 5 colonies of the streptococcus. Control cultures from the adjacent normal mucous membrane were negative except for a few colonies of staphylococci and aerobic saprophytic bacilli.

Nov. 4.—Subcultures of 8 colonies of the small gram-positive streptococcus from the ulcer and of 3 of those from the node, yielded small grayish non-hemolyzing nonadherent colonies on blood-agar plates, and short chains in ascites dextrose broth identical with those found in Case 773 (Fig. 1).

The streptococcus in second culture was injected into 1 rabbit and 1 dog. The mucous membrane of the rabbit did not show lesions at the end of 1 week, while that of the dog, 4 months after the injection, showed a scar indi-

cating a healed ulcer near the pyloric ring. After the streptococcus had undergone 1 animal passage, the following experiment was made.

*Dog 42.*—Injected intravenously, Nov. 7, with the growth from 150 c.c. of an ascites-dextrose-broth culture of the streptococcus isolated from the blood of the dog mentioned.

Nov. 8.—III, lay quiet, did not eat. There was no tenderness or swelling of the joints.

Nov. 11.—III. Chloroformed, and examined at once. In the stomach there were numerous small (1 to 5 mm.) and large (1 to 3 cm.) hemorrhagic areas, areas of necrosis, erosions, and ulcerations (Fig. 3). The lesions were more numerous in the fundus, but larger in the pylorus. The intervening mucous membrane, except for slight congestion, was normal, as was that of the duodenum. The stomach contents, free from food, were strongly acid in reaction and contained a small amount of altered blood. The mucous membrane of the small and large intestines was congested.

Sections through two areas of hemorrhage and ulceration in the cardiac end of the stomach showed marked extravasation of blood, which was most marked as the surface of the mucous membrane was reached. This was true in practically all the areas of hemorrhage. In one there was distinct ulceration. The ulcerated area was covered in places by a thick layer of necrotic cells, numerous leukocytes, blood corpuscles, and fibrin. The mucous membrane beneath this layer presented an interesting picture. There were, first, a dense layer of polymorphonuclear leukocytes, then a layer in which there was marked hemorrhage with less leukocytic infiltration, and then the more normal gland structure, throughout which were found leukocytes in large number (Fig. 4). The chief cells showed marked degeneration; the protoplasm was granular, the nuclei fragmented, with marked desquamation. The eosin-staining parietal cells, on the other hand, appeared normal, even where all the chief cells had disintegrated and desquamated. Some of these retained normal staining properties and position, even where they had been detached from the acini (Fig. 5). The other coats were unchanged. The blood vessels in the submucosa were dilated and a number of veins showed rather marked mural implantation of leukocytes, appearing as a beginning thrombosis (Fig. 4). Search for bacteria revealed few diplococci in the deeper layers, but masses of diplococci and short chains beneath the sloughing necrotic layers, as is well shown in Fig. 6. On the surface were a few large gram-staining bacilli, but none in the deeper layers.

After 2 animal passages the streptococcus was injected into 3 rabbits and 1 dog. One rabbit died in 24 hours with streptococcemia, hemorrhage in the septum of the heart, and acute splenitis, but without lesions in the stomach. The other two died in 4 and 18 days. The one dying in 4 days showed multiple arthritis, the other arthritis of the right knee, and a healing ulcer (5 by 3 mm.) in the cardiac end of the stomach. The dog which died in 18 days had developed 2 healed ulcers of the cardiac end of the stomach, a local nephritis, fatty degeneration of the liver, and localized myocarditis.

After 3 animal passages the streptococcus was injected into 1 dog, which died from an overwhelming infection in 24 hours. The staphylococcus was repeatedly injected intravenously into 2 rabbits, neither of which developed lesions of the stomach. Mixtures of the cultures of the staphylococcus and the streptococcus fed with chopped meat and sharp particles of dried splintered bone to 2 dogs, did not produce lesions.

## CASE B

Ulcer of the stomach in a woman 28 years of age, whose symptoms had existed for 5 years. This ulcer, which was 3 cm. from the pyloric ring, was adherent to the duodenum and the pancreas, causing hour-glass constriction.

The usual streptococcus was isolated in pure culture, and injected as soon as sufficient growth could be obtained, into 2 rabbits and 3 dogs of medium size.

The rabbits died in 24 hours. They were examined immediately. Both disclosed marked, but localized, hemorrhages of the stomach with gaseous distention. One showed, in addition, hemorrhages in the pericardium and in the aortic valve, while the other showed small hemorrhages in the tricuspid valve. Cultures from emulsions of the hemorrhagic areas in the stomach yielded many streptococci; those from the blood, a smaller number.

All 3 dogs had developed lesions of the stomach. The one which had received the largest dose died in 24 hours, showing acute dilatation of the stomach, with circumscribed hemorrhages in the cardiac end, a large amount of brownish fluid, no food, and gaseous distention. The gas in the stomach of the dog, as in both the rabbits, contained large amounts of carbon dioxide. There were, in addition, hemorrhages of the mucous membrane of the colon. Another dog died in 48 hours, with one acute ulcer of the fundus extending through the mucous membrane, numerous small punctate hemorrhages, some of which showed beginning ulceration, and numerous small and occasionally white necrotic areas in the mucous membrane of the large intestine. The third dog, chloroformed 8 days after injection, had developed a small round ulcer (5 mm. in diameter) of the pylorus, 2.5 cm. from the pyloric ring. Cultures from the blood were sterile, while those from the ulcer gave a moderate number of nonhemolyzing streptococci.

The strain isolated from the dog which died in 4 hours (1 animal passage) was injected into 2 rabbits and 2 cats. One rabbit died in 2 days, the other in 13 days. The former showed hemorrhage in the tricuspid valve with a beginning endocarditis, the latter what appeared as a phlegmonous gastritis, cholecystitis, medullar nephritis and pyelitis, and endocarditis. Cultures from the mucous membrane of the second rabbit yielded a large number of streptococci and a few colonies of colon bacilli. The blood was sterile.

The cat which had received the large dose showed after 24 hours marked gaseous (carbon dioxide) distention of the stomach, extensive hemorrhage of the mucous membrane of the fundus, and a moderate amount of chocolate-colored blood. Cultures from the blood gave 20 colonies; from the spleen, 40; two areas of hemorrhage in the mucous membrane yielded 2,000 and 3,000 colonies, and the adjacent normal mucous membrane, 40 colonies of the non-hemolyzing streptococcus.

The cat which had received the smaller dose seemed ill, ate poorly, and lost in weight for a number of weeks, then regained weight and became apparently perfectly well. It was chloroformed 14 weeks after injection. No lesions were found except a scar (0.5 cm. in diameter) in the mucous membrane of the stomach, 2 cm. from the pyloric ring.

## CASE 885

Chronic ulcer of the stomach with perforation. An emergency operation was performed Jan. 31, 1914, by Dr. Hirst. There were a perforating ulcer of the stomach and a beginning peritonitis. The ulcer was excised and the area inverted. The patient recovered.

Cultures yielded approximately 5,000 colonies of the usual nonhemolyzing streptococcus and a few colonies of staphylococci and sarcinae.

*Dog 65.*—Injected intravenously, Feb. 4, with the growth from 150 c.c. of an ascites dextrose broth of the streptococcus in its second culture.

Feb. 14.—Seemed ill, ate little, and had lost in weight.

Feb. 22.—Found dead. Stomach was distended with gas rich in carbon dioxid. It contained a small amount of brown blood-tinged fluid, but no food. A number of small deep ulcers were found in the fundus. In two of these, the inflammation extended through to the peritoneal coat, which was opaque and hyperemic. The mucous membrane of the pylorus was normal. The duodenum showed one large ulcer (1 by 2.5 cm.). The peritoneal coat here was adherent to the surrounding structures and formed the floor of the ulcer.

Sections through one of the ulcers in the stomach revealed leukocytic infiltration in the margin and in the peritoneal and subperitoneal layers, and a moderate number of diplococci. The muscular coat, however, was quite free from infiltration.

#### CASE 52

Ulcer of the stomach and duodenum in a woman 62 years of age. The patient had had for years severe attacks of migraine. Three years previously she had had a severe attack of herpes zoster, involving the left thorax, and 2 years previously there had been a recurrence of severe pain in the zoster area, but no blistering. One year later she had had two severe hemorrhages from the stomach, but had made a good recovery under medical management, and was again quite free from gastric symptoms. At this time marked tartar deposit was found about the teeth, and marked gingivitis, especially at the outer aspect of the second right lower molar. The patient was advised to have her teeth put in order, but this was not done.

On Aug. 19, 1914, she began vomiting large quantities of blood on two occasions several weeks after an attack of so-called grippe. Three days after the hemorrhages from the stomach, the temperature rose. Altho there were no physical indications of pneumonia or other demonstrable cause, a high fever continued until death occurred.

Only a partial postmortem examination was made. The heart showed chronic mitral endocarditis. The lungs were edematous, but there was no pneumonia. The wall of the duodenum just beyond the pylorus showed two thickened puckered areas, which produced a marked sacculation on the anterior wall, approximately 2.5 cm. in diameter. On opening the stomach, there were found approximately 100 c.c. of mucopurulent material, free from food. The puckered areas in the duodenum were scars of healed ulcers. Just outside of the pyloric ring on the posterior wall, there was an indurated, partially healed ulcer, 0.4 by 1 cm., having a hemorrhagic base. In the pyloric third of the stomach, there were an entirely healed ulcer, and one acute ulcer having a hemorrhagic base, which was not thickened. The mucous membrane of the entire stomach was hyperemic, in places edematous, and studded with numerous punctate hemorrhages, the membrane over some of the hemorrhagic areas being eroded. The mucous membrane adjacent to the ulcer was no more hyperemic than the rest. The lymph glands in the gastro-colic omentum were enlarged and hemorrhagic on the cut surface. No thrombosed blood vessels in the stomach could be found. The gall-bladder was distended with a dark bile, the ducts patent. No gall-stones. The liver showed marked granular and fatty degeneration.

The stomach contents did not have an acid odor and were only slightly acid to litmus. No peritonitis.

Cultures were made from the blood, lymph gland, liver, duodenal contents, duodenal and gastric ulcers, and from pus obtained from the depths of the inflamed gum covering the deposit of tartar on the right lower molar.

Aug. 31.—Cultures in ascites dextrose broth from the gum gave a pure culture of a short-chained streptococcus (Fig. 2); those from the duodenal contents, the emulsion of the ulcers, the blood, the lymph gland, and the liver, gave streptococci and colon bacilli. Ascites-dextrose-agar shake cultures from the ulcer emulsion yielded approximately 6,000 colonies of streptococci, and approximately 150 colonies of colon bacilli. The material withdrawn with a pipet from the margin of the duodenal ulcer—the surface having first been seared—showed mostly streptococci, but also a few colon bacilli.

Sections of the ulcer in the duodenum and of that in the stomach disclosed hemorrhage and leukocytic infiltration of the margin and base, extending through the entire thickness of the mucosa and submucosa. The muscular coat was largely replaced by connective tissue in the case of the duodenal ulcer, but not in the case of the gastric; in the latter was marked leukocytic infiltration of the peritoneal coat, but no perforation. Sections through one of the edematous and hemorrhagic areas revealed marked dilatation of the intra-glandular blood vessels, hemorrhage, and leukocytic infiltration. Gram-Weigert stains of sections of the ulcers disclosed numerous streptococci in the margins and bases of the ulcers, and in the peritoneal coat. Those of the edematous areas also showed a number of streptococci. On the surface of the ulcers, as of the hemorrhagic areas, there were found scattered bacilli.

The streptococcus from the tooth was injected, in the first culture, into 4 animals. Of these, all but one showed hemorrhage or ulcer of the stomach. The results in the case of the dog are given in the following.

*Dog 120.*—Injected intravenously Sept. 1, 1914, with the growth from 150 c.c. of ascites dextrose broth of the streptococcus from the tooth.

Sept. 3.—Dog seemed well. Chloroformed. The mucous membrane of the pyloric end of the stomach was studded with numerous small punctate hemorrhages. There were present a number of larger hemorrhages and 4 ulcers, from 2 to 4 mm. in diameter, near the junction of the middle and lower thirds of the stomach. The intervening mucous membrane was normal. The gastric contents, which had a typical odor, were highly acid in reaction. The gall-bladder, appendix, pancreas, and other organs, appeared normal.

Sept. 4.—Cultures from blood, bile, liver, and joint fluid, sterile. Cultures from the larger ulcer yielded 150 colonies of streptococci, those from the small ulcer 80 colonies, while those from a hemorrhagic area without ulceration of corresponding size, gave approximately 2,400 colonies of the injected streptococci.

The streptococcus from the ulcer was injected, in its second culture, into 12 animals. Of these 8 developed lesions in the stomach, and 5 lesions in the gall-bladder.

After 1 and 2 animal passages, the streptococcus was injected into 6 more animals, giving rise to lesions in the stomach in 1, and to lesions in the gall-bladder in 3.

#### CASE 112

Single woman, 51 years of age; bookkeeper. She had complained of pain and other symptoms in the epigastrium typical of duodenal ulcer, for 1 year. Pain had been especially severe 6 weeks prior to examination. Aug. 18, 1914,

there were found by Dr. Judd a subacute ulcer of the duodenum, 1 cm. beyond the pylorus, and a chronically inflamed adherent appendix. The overlying and adjacent visceral and parietal peritoneum was very red, loosely adherent, and edematous. The location of the ulcer and the severity of the accompanying inflammation precluded excision of the ulcer. After covering the ulcer with omentum and partially occluding the pylorus, posterior gastro-enterostomy and appendectomy were made. The patient made a prompt recovery.

Sept. 3.—Cultures from the thickened and injected visceral peritoneum overlying the ulcer, gave a moderate number of short-chained nonhemolyzing streptococci and a few colonies of a gram-staining bacillus resembling *B. subtilis*, while the parietal peritoneum adjacent to the ulcer yielded a few colonies of the same streptococci in pure growth. The sections of the thickened visceral peritoneum showed marked fibrous thickening, perivascular round-cell and leukocytic infiltration, and hemorrhages. Diplococci were found, two of which are shown in Fig. 8.<sup>41</sup>

The streptococcus in its second culture was injected into 4 dogs, 2 rabbits, and 1 guinea-pig, all of which developed ulcer. All the dogs, 1 rabbit, and the guinea-pig developed duodenal ulcer; the other rabbit, ulcer of the stomach and cholecystitis. The dogs, in addition to ulcer of the duodenum, had marked hemorrhages in the stomach and the duodenum. Three of the 4 dogs showed a peritonitis of the lesser peritoneum surrounding the duodenal ulceration. In the dog which developed localized hemorrhages in the gall-bladder, the peritoneal exudate had extended to the gall-bladder and to the under surface of the liver.

After 1 animal passage the strain was injected into 2 dogs. One developed 2 deep hemorrhagic ulcers in the duodenum, hemorrhages of the stomach, marked cholecystitis, localized peritonitis, and hepatitis; the other, cholecystitis and hemorrhages in the duodenum, but no ulcer.

After 2 animal passages the streptococcus was injected into 3 dogs and 2 rabbits. One dog developed an ulcer in the pyloric end of the stomach, endocarditis, and infarcts of the kidney; one, cholecystitis and focal nephritis; the third, ulcer of the stomach and of the duodenum. One rabbit gave ulcer of the stomach, and the other ulcer of the duodenum.

*Dog 134.*—Injected intravenously, Nov. 4, 1914, 78 days after the original cultures had been made, with the growth from 60 c.c. of an ascites dextrose broth of the streptococcus from the ulcer in Case 112.

Nov. 5.—Found dead. The mucous membrane of the stomach was hyperemic, with numerous small hemorrhages, especially in areas in the pyloric end. The first portion of the duodenum, which was markedly hyperemic, contained three hemorrhagic areas from 5 to 6 cm. in diameter. The mucous membrane in the center of one of these areas just outside the pyloric ring was necrotic and ulcerated. The rest of the mucous membrane of the duodenum and intestine was normal, except for small hemorrhages, especially in the lymph follicles. The peritoneum of the upper portion of the small intestine was hyperemic and opaque, while over the duodenum, gallbladder, and the under surface of the liver, there was a thin layer of loosely adherent fibrinous exudate. The mucous membrane of the gall-bladder was normal. There was a pea-sized hemorrhagic lymph gland adjacent to the duodenum. The mesenteric glands were normal. No other gross lesions.

Nov. 7.—Cultures from the crushed area of hemorrhage in the duodenum yielded fully 10,000 colonies of streptococci, and only 12 colonies of colon bacilli, and the blood a moderate number of gray-producing nonhemolyzing colonies of streptococci. The bile and peritoneal exudate revealed a large number of streptococci in pure growth. The joint fluid was sterile.

*Dog 142.*—Injected intravenously Sept. 11, 1914, with the growth from 75 c.c. of an ascites-dextrose-broth culture of Strain 112 after 1 animal passage.

Sept. 12.—Dead. There was marked hemorrhage of the cardiac end of the stomach, of the duodenum, and of the small intestines. The gall-bladder was markedly hemorrhagic and edematous, especially over the fundus, where the wall measured from 0.4 to 0.6 cm. The edema was most marked in the submucosa. Mucous membrane swollen, but not ulcerated. Fluid expressed from the wall of the gall-bladder and the surrounding structures, bile-stained. Walls of the cystic and common ducts also edematous. Lymph glands adjacent to the common duct, hemorrhagic. Except for subendocardial hemorrhages, particularly of the left ventricle, no other noteworthy lesions found. Smears from the edematous fluid from the wall of the gall-bladder gave a moderate number of streptococci.

Sept. 13.—Blood-agar-plate cultures of the blood yielded 50, of the bile 2,500, and of the wall of the gall-bladder 8,000 colonies of nonhemolyzing streptococci. The bile showed, in addition, 5 colonies of colon bacilli. Cultures from the bile in tall columns of ascites dextrose agar gave streptococci, gas bacilli, and colon bacilli; from the wall of the gall-bladder, streptococci and gas bacilli, and from the liver, a few colonies of streptococci.

*Dog 156.*—Injected Sept. 19, 24, and Oct. 2, with the growth from 20, 30, and 40 c.c., respectively, of an ascites dextrose tissue broth of Strain 112, after 2 animal passages.

Oct. 10.—Seemed ill and very weak. Chloroformed, and examined at once. Mucous membrane and tissues everywhere pale, the blood showing only 50% hemoglobin. The stomach, free from food, contained a small amount of brownish material, resembling altered blood. The mucous membrane of the stomach contained approximately 10 small erosions, surrounded by whitish necrotic swollen areas, and a number of small ulcers filled with adherent brownish blood clots. There was one large deep ulcer, 0.6 by 1 cm., in the duodenum just above the ampulla of Vater (Fig. 10). The margin was edematous and necrotic and the base was filled with clotted blood. The liver showed marked fatty degeneration. The lumen of the small and large intestines contained a moderate amount of partially digested blood. No other noteworthy lesions. Smears from the necrotic margin of the ulcer in the duodenum revealed many leukocytes, gram-staining diplococci, and short chains.

Oct. 15.—Cultures from the blood yielded nonhemolyzing streptococci, and from the bile, gas bacilli. Sections through the base of the ulcer showed in the center complete absence of mucous membrane and submucosa, and necrosis of one-third of the circular layer of the muscular coat. There was leukocytic infiltration between the disintegrating epithelial cells in the submucosa, chiefly around vessels, along the connective-tissue stroma, between muscle bundles, and beneath and in the thickened and adherent peritoneal coat. There was no extravasation of red blood corpuscles. The portion of the base of the ulcer which had not yet entirely sloughed, was composed of poorly staining connective-tissue stroma, in which were fragmented cells, leukocytes, thrombosed vessels running at right angles to the floor of the ulcer, and 2 large thrombosed vessels in the submucosa (Fig. 11). The thrombi, which were partially organized,

contained a moderate number of leukocytes. In several sections of a large series studied, there was marked leukocytic infiltration surrounding the thrombosed vessel in the submucosa. Gram-Weigert stains showed a moderate number of diplococci, chiefly in the area of leukocytic infiltration, and a few in one of the thrombi in the small vessels shown in Fig. 12. On the surface of the ulcerated area and the adjacent normal mucous membrane, there were found a few scattered cocci and large bacilli.

#### CASE 236

Recurring ulcer of the stomach in a young man. I am indebted to Dr. Sippy for this case. The patient had developed typical symptoms of ulcer of the stomach 6 months before, after ulceration of a tooth. He had completely recovered under medical management. Since that time, he had had a discharging sinus, as a result of trouble with the tooth, which alternately healed, formed a blister, and then again discharged pus. There had been a sudden recurrence of the ulcer of the stomach associated with hemorrhage, 5 weeks prior to examination.

On Jan. 22, 1915, two days previous to the extraction of the tooth, a culture was made from pus withdrawn from the sinus after sterilization of the surface and insertion of a pipet for a depth of 1 cm. A blood-agar-plate culture gave an almost pure culture of *Streptococcus viridans*, with a few colonies of hemolytic streptococci. Ascites-dextrose-broth cultures yielded a pure growth of a short-chained streptococcus. The cultures from the tooth pulp, 2 days later, yielded exactly similar results.

Injection of this strain into guinea-pigs, rabbits, and dogs, showed a most pronounced tendency on the part of the organism to lodge in the mucous membrane of the stomach, producing hemorrhages and ulceration. Intravenous injection of the strain from the sinus was made into 2 rabbits and 1 dog. The dog developed ulcer in the duodenum; one rabbit gave hemorrhage and ulcer of the cardiac end of the stomach, and the other rabbit arthritis, but no lesions of the stomach.

The cultures from one of the ulcers in the dog gave the streptococcus in pure growth in broth and in ascites dextrose agar (17 colonies). The broth culture, injected intravenously into 1 rabbit and 1 guinea-pig, produced hemorrhage or ulcer in both; injected intraperitoneally into 1 rabbit and 1 guinea-pig, it produced in the rabbit an acute ulcer (2 by 3 cm.) in the lesser curvature of the stomach, but no lesions in the guinea-pig; injected intrapleurally into 1 guinea-pig, it caused two small ulcers in the pyloric end of the stomach; injected subcutaneously into 1 guinea-pig, it gave rise to no lesions in the stomach. The streptococcus culture from the tooth was injected intravenously into 1 rabbit and 1 dog, and intraperitoneally into 1 guinea-pig. All developed hemorrhage and ulcer of the stomach or of the duodenum. Cultures from one ulcer from each of 7 animals yielded from 5 to 40 colonies of streptococci, irrespective of whether the injection had been made intravenously, intraperitoneally, or intrapleurally.

#### CASE 227

An ulcer of the stomach in a physician of middle age. There was a history of severe recurring attacks of tonsillitis and rhinitis for years. On April 21, 1914, during an attack of acute rhinitis and acute indigestion, resembling ulcer, there was isolated a pure culture of a green-producing streptococcus from the mucopurulent discharge from the nose. This was injected into 5 animals. Of

these, 2 developed ulcer of the stomach, 1 hemorrhages of the stomach, and 2 cholecystitis. No particular importance was attached to these findings at that time.

Dec. 19.—The symptoms of gastric ulcer were typical. The tonsils showing marked infection, tonsillectomy was done. Salt-solution suspensions of the extirpated and washed tonsils caused hemorrhage or ulcer of the stomach in 2 rabbits. Cultures from the tonsils, injected into 6 animals, produced hemorrhages or ulcer of the stomach in 4.

After the removal of the tonsils, while the patient was being treated with an autogenous vaccine prepared from the streptococcus isolated from the ulcer in one of the animals, the symptoms of ulcer were reduced for a short time, but they returned, and persisted in spite of strict medical management (Dr. Frick).

Feb. 27.—The teeth showed the presence of a number of pyorrheal pockets. Cultures from the pus from these pockets injected into 1 dog produced hemorrhage and ulcer of the stomach. On the basis of these findings, the involved teeth were extracted on Aug. 15, 1915, the symptoms of ulcer having persisted during the interval. He was then placed on strict medical treatment for ulcer, and has since been free from symptoms and has regained his former weight.

#### CASE 531

Recurring ulcer of the stomach in a woman 34 years of age (Dr. Plummer). The trouble had begun 8 years before, with recurring attacks of sharp cramp-like pain in the epigastrium. The attacks were usually associated with nausea, in former years with vomiting and numerous hemorrhages from the stomach, and were followed by soreness in the epigastrium. The attacks, which always followed tonsillitis, lasted from 10 to 14 days and were followed by intervals of 3 or 4 months of almost complete relief. Systolic blood pressure 122, temperature 99.2, hemoglobin 70%, urine normal, except for a small amount of albumin. A test meal showed a total acidity of 48, free hydrochloric acid 28, combined acids 20, and no occult blood. An x-ray of the chest and stomach was negative. Tonsils small and only moderately infected. Slight tenderness over the right abdomen, most marked over the right lower quadrant, but no muscle spasm. On account of the apparent etiologic relation between the tonsils and her attacks, tonsillectomy was advised. The extirpated tonsils, which were small, revealed only a moderate grade of infection.

Cultures gave chiefly *Streptococcus viridans*, a few hemolytic streptococci, and staphylococci. The broth cultures for injection yielded chiefly rather long-chained streptococci.

These cultures were injected intravenously into 2 guinea-pigs and 4 rabbits. All but 1 rabbit and 1 guinea-pig showed either hemorrhage or ulcer, or both, of the stomach or of the duodenum. The rabbit injected with 3 c.c. of the emulsion from the tonsils developed both hemorrhage and ulcer of the stomach. Only 2 of the animals died. The rest seemed well. An ulcer which was cultured, yielded 50 colonies of streptococci in pure growth. Since the tonsillectomy, the patient has gone for a longer interval without gastric attack than at any time during a period of 8 years.

In previous papers,<sup>40, 43</sup> I have pointed out that when strains of streptococci of low virulence are passed successively through animals, their place of localization changes with returning virulence, and that

when these strains reach the stage at which they give rise to muscle lesions, they are apt also to produce ulcer of the stomach and focal nephritis. The following experiment will serve to illustrate these observations:

*Dog 25.*—Injected intravenously March 20, 1913, with the growth from 240 c.c. of an ascites-dextrose-broth culture of streptococcus (R51A<sup>30</sup>) isolated 11 years previously as a pneumococcus in pneumonia. It had lost practically all virulence years before, had acquired hemolytic properties, and was now in the 20th animal passage.

March 27.—The dog was lame in left front leg.

March 28.—Turbid fluid from both knee joints.

March 29.—Right wrist was swollen and tender; the animal limped, was sensitive over muscles; movements caused pain.

April 8.—Found dead, body warm. Marked pallor of all tissues; hemoglobin 40%. Moderate amount of altered blood in stomach. There were one large ulcer (1 by 3 cm.) in the duodenum, and one smaller ulcer (0.5 by 1 cm.) in the pyloric ring, while the rest of the stomach showed areas of hemorrhage and small ulcers. The margins of the ulcers, which were infiltrated, appeared necrotic. The ulceration in the duodenum had extended to the peritoneal coat, where there were peritoneal adhesions. The muscles contained numerous whitish streaks, especially the superficial muscles of the neck, shoulders, thorax, and diaphragm. The myocardium, which was flabby, contained a moderate number of whitish areas. Similar lesions were found in the nonstriated muscle fibers of the large and small intestines. The kidneys showed the picture of focal and ascending nephritis. There was present also suppurative conjunctivitis of the right eye, associated with hemorrhages of the sclera at the limbus.

Sections of the ulcer of the pyloric end of the stomach showed marked leukocytic infiltration and hemorrhage in its base, degeneration of the mucous glands, and beginning fibrosis (Fig. 22). Stains for bacteria showed a moderate number of diplococci in the submucosa and in the ulcerated area.

## RESULTS

The details of these experiments suffice to illustrate the results obtained.

The injection of staphylococci and of *Bacillus subtilis* from 4, and of the yeasts from 1 of the ulcers isolated in the earlier cases, produced no lesions in the stomach, and hence were considered accidental invaders.

Cultures of streptococci which in the earlier experiments had proved able to produce ulcer when injected intravenously, failed to do so when injected into dogs fed with mixtures of meat and sharp particles of bone.

In Table 1 is given a summary of all the results following intravenous injection of streptococci from ulcer. Hemorrhage and ulcer of the mucous membrane of the stomach or of the duodenum occurred

following injection of 19 strains when first isolated in 61% and 60% respectively of 117 animals injected. This is in marked contrast to an incidence of 20% hemorrhages and 8% ulcers following injection of 180 strains of streptococci from sources other than ulcer.

It should be emphasized here that these results were not obtained during a short interval only, but that the experiments extended over a period of 3 years, were done in different localities, and at all seasons of the year. They included altogether 6 species of animals (dog, rabbit, guinea-pig, monkey, cat, and mouse).

The selective affinity for the stomach and the duodenum disappeared both after cultivation of the bacteria on artificial media for from 1 to 6 weeks and after animal passage, the incidence of ulcer dropping from 60% to 0% after cultivation and to 33% after animal passage. The strains kept on artificial media acquired greater affinity for the

TABLE 1  
ELECTIVE LOCALIZATION OF STREPTOCOCCI FROM ULCER OF THE STOMACH

The Time of the Injection of Streptococci	Strains	Animals Injected	Percentage of Animals Showing Lesions in		
			Appen- dix	Stomach and Duodenum	
				Hemor- rhage	Ulcer
When isolated.....	23	117	2	61	60
Later.....	8	22	5	5	0
After animal passage.....	7	39	0	23	33

appendix. The strains passed through animals acquired greater affinity for the gall-bladder and the pancreas.<sup>43</sup> While the affinity for the stomach was usually more marked in the streptococci from the ulcer itself than in those from the focus of infection, yet the latter had an unmistakable affinity also. In the case of recurring ulcer of the somach (Case 236) following an ulcerated tooth, the streptococcus from the pus from the sinus showed an affinity for the stomach so marked that it produced ulcer not only after intravenous, but also after intraperitoneal and intrapleural, injections.

In the case of recurring ulcer (Case 531) in which the attacks always followed tonsillitis, ulcer of the stomach followed injection of the bacteria directly from the extirpated tonsils and of the primary cultures from the tonsils, in all but 2 of 7 animals injected. Streptococci from the alveolar abscess at the apex of an extracted tooth in a patient with acute ulcer of the stomach and gastric hemorrhage, produced, on

intravenous injection, hemorrhage and ulceration of the stomach or of the duodenum in 2 dogs and in 1 of 2 rabbits injected. In another case of acute ulcer following an attack of grippe, cultures from the tonsil and from the infected area about the left lower wisdom tooth, produced a hemorrhagic ulcer in the lesser curvature 2 cm. from the pyloric ring in 2 rabbits, both of which seemed well at the time they were chloroformed, 72 hours after injection.

In some instances the strains from duodenal ulcers showed an unmistakable tendency to produce ulcer in the duodenum oftener than in the stomach (see Cases 773 and 112). The strain from the peritoneal coat of the ulcer in Case 112, not only showed this to a striking degree, but in a number of animals, the infection perforated the wall of the duodenum, and produced a localized peritonitis like that present in the patient. Some of the strains from gastric ulcer tended to pro-

TABLE 1—*Continued*  
ELECTIVE LOCALIZATION OF STREPTOCOCCI FROM ULCER OF THE STOMACH

Percentage of Animals Showing Lesions in									
Gall-bladder	Pan-creas	Intes-tines	Joints	Endo-cardi-um	Peri-cardi-um	Myo-cardi-um	Muscles	Kidney	Lung
20	3	7	16	12	4	5	0	5	0
5	0	0	15	14	0	0	0	0	0
30	15	15	21	5	0	3	3	5	15

duce ulcer in the stomach oftener than in the duodenum (see Case 779). This selective localization occurred too often to be accidental. In other instances, no such definite relationship between the place of isolation and the place of localization was shown.

Either or both hemorrhage and ulcer of the stomach or of the duodenum followed injection of all the strains from ulcer in a total of 93 animals (83%). Hemorrhage occurred in the pyloric portion in 38 instances, in the fundus in 34, and in the duodenum in 20; ulcer occurred in the pyloric portion in 28, in the fundus in 32, and in the duodenum in 14. Either or both hemorrhage and ulcer followed injection of strains of streptococci from sources other than ulcer, in a total of 99 animals (26%). In these, hemorrhage occurred in the pyloric portion in 38 instances, in the fundus in 29, and in the duodenum in 27; ulcer occurred in the pyloric portion in 24, in the fundus in 14, and in the duodenum in 7. The location of the ulcer corre-

sponds to the location of the hemorrhage and the location of both lesions following injection of streptococci from ulcer and other sources is comparatively fixed, being most often in the pyloric portion, along the lesser curvature, or in the duodenum, and least often in the fundus of the stomach.

In summarizing the locations of 25 ulcers, which were disclosed from 1 to 17 weeks after the primary injection, many of which, chiefly in dogs, had taken on features typical of chronic ulcer, it was found that 12 were in the pyloric portion, 4 being along the lesser curvature, 2 in the fundus, and 7 in the duodenum. Healing ulcer, or scars of healed ulcers, were found in 7 instances, from 18 to 120 days after injections. All but 2 of these were situated in the fundus, the others in the pyloric portion.

The location of the experimental ulcers, therefore, corresponds strikingly to the location of ulcer in man, as found at autopsy and in the operating room (Mayo<sup>44</sup>). The duodenal ulcers occurred usually just outside (Fig. 16), and always within two inches of, the pyloric ring. In the dogs, they were found chiefly in the anterior and posterior walls. Hemorrhage and ulceration at the ampulla occurred oftener in the rabbits than in the dogs. In one such ulcer, the orifice was plugged with mucus, and the common duct was edematous.

Severe or fatal hemorrhage occurred from the more chronic ulcers in 7 instances. Three of the ulcers were situated in the pyloric portion of the stomach and 4 in the duodenum. Four of these showed thrombosis of blood vessels in the submucosa.

In some cases, the anemia was out of all proportion to the amount of blood found in the stomach and bowel at autopsy. This indicated that repeated hemorrhages had occurred, or that a slight oozing had persisted for a long time.

The incidence of ulcer following injection of the streptococci from cholecystitis (15%), acute appendicitis (1%), rheumatic fever (18%), herpes zoster (8%), and other sources, corresponds in a general way to the incidence of ulcer in those diseases in man.

The chief difference between the lesions in the stomach or the duodenum following injection of the strains from ulcer, and those following injection of strains from other sources, was one of total incidence and degree, rather than of kind, the strains from ulcer showing by far the greater affinity for the stomach and duodenum. The lesions in both cases were due to localized infection of the mucous membrane, usually demonstrable at the time of examination.

The results given in the table were obtained without special attention to the time of injection of the bacteria in relation to the functional activity of the stomach. The fact that in a given series of animals injected with the same strain, there were one or two in which the stomach showed no lesions whatever when the stomach in all the rest showed marked lesions, indicates that the state of function is of importance in determining the localization and production of ulcer.

Colon bacilli were almost always absent in both duodenal and gastric ulcer in animals which were chloroformed. This is in accord with the fact that colon bacilli were absent in nearly all the ulcers in man excised at operation.<sup>41</sup>

#### DESCRIPTION OF THE ULCERS

In most instances, there occurs a primary lodgement and growth of bacteria, in the interstitial tissue of the glands and between the cells, followed by hemorrhage, necrosis, and ulceration. The loss of tissue usually begins in the center of the hemorrhagic infiltrated and necrotic area as early as 18 hours after injection, and spreads to the periphery. The surrounding blood vessels are congested. In some instances, the ulcer forms without a preceding hemorrhage, in circumscribed grayish swollen necrotic areas. The ulcers are deeper in the center and penetrate rapidly to the muscularis mucosa. The base in the acute ulcer is hemorrhagic, while in the more chronic type, it is clean.

Microscopically, both the circumscribed hemorrhage and the ulcer are cone-shaped, with the base of the cone at the surface and the apex at the muscularis mucosa. The streptococci lodge in the fine capillary network about the gland tubules or tissue spaces, and multiply so that in 24 or 48 hours there are often enormous numbers of streptococci at the apex of a hemorrhagic area (Figs. 15, 18, and 20). A necrotic process surrounds this area, and as the overlying mucous membrane sloughs, it carries with it most of the bacteria (Fig. 6.)

This finding explains the observations made repeatedly; namely, that the cultures from the ulcerated area often show fewer colonies than those from adjoining areas of hemorrhage (experiment on Dog 120 with streptococcus from the tooth in Case 52). The number of colonies, especially if the animal has been dead for a time, is often surprisingly small, ranging usually from relatively few to thousands. From a careful study of numerous sections and of the results of the cultures, it is certain that following the primary sloughing, the number

of streptococci in the remaining more or less healthy tissue is not usually very large, bacteria being often hard to find.

The cellular infiltration surrounding the chronic ulcers is usually not marked, but frequently extends into the submucosa, the muscular layer, and at times through the peritoneal coat. The connective-tissue formation is well advanced in some ulcers and at times extends far beyond the ulcerated area (Fig. 22). In some instances, it involves the muscular layer and peritoneal coat (Fig. 8). In the chronic ulcers, both in man and in animals, the streptococci present are few in number and are found chiefly in the areas showing cellular infiltration surrounding the necrotic process, along the supporting tissue, the gland tubules, along partition membranes, and not infrequently in the sub-peritoneum. There are often marked intraglandular infiltration, disintegration, and disappearance of the chief cells of the gastric tubules while the parietal cells remain unaffected (Fig. 5). This is in agreement with the findings of Korczynski and Jaworski<sup>45</sup> in the case of ulcer in man. Completely or partially thrombosed blood vessels have been found in or adjoining the experimentally produced ulcers in 9 instances. In 3 of these, the thrombi contained streptococci (Dog 156, Case 112, and Figs. 11 and 12).

In 4 instances only, the ulcer appeared to be secondary to a primary infection in the lymph follicles, but in none of these was the evidence entirely convincing (Fig. 23).

#### THE MECHANISM OF THE FORMATION OF THE ULCER

Bensley and Harvey<sup>46</sup> have shown that the hydrochloric acid in the normal stomach is formed on the free surface. It occurred to me that possibly the localized infection might alter the secretory activity of the cells, and that the hydrochloric acid might be formed within the cells in the depths of the mucous membrane, which would then directly become digested and the ulcer result. An attempt was therefore made to study this point by the use of indicators under the conditions given by Bensley and Harvey. I wish here to express my appreciation to Dr. Harvey for valuable aid and suggestions in the use of their technic.

The difficulties to be overcome were obviously great. The affinity of many strains for the stomach is transient. The size of the injection had to be regulated so that the amount of ulceration and general illness would not be sufficient to interfere with the appetite of the animal. The lesion had to be in the acid-secreting portion. The process had to be studied after distinct lesions had taken place, but preferably

before marked ulceration had occurred. Repeated experiments resulted in failures, but in 2 rabbits and 1 dog all necessary requirements were fulfilled. I cite only one experiment.

*Rabbit 676.*—Injected intravenously June 28, 1914, with the growth from 45 c.c. of an ascites dextrose tissue broth of a streptococcus (31) from ulcer in man.

June 29.—Seemed well, was fed cabbage and grass, ate heartily; killed by sudden blow on the head. Stomach removed and opened at once. The content was highly acid to litmus. The mucous membrane was washed for an instant in cold water. There was a hemorrhage with beginning ulceration in the mucous membrane of the cardiac end of the stomach (3 by 2 mm.). One-half of the ulcer was saved for cultures, the other studied at once. Bits of the hemorrhagic mucous membrane with beginning ulceration in the center, and adjoining normal mucous membrane were placed in saturated solution of cyanamin bichlorid, in salt solution, and in a solution of 1 part of neutral red to 10,000 parts of salt solution, where they were allowed to remain for a few minutes. They were then rapidly mounted on slides in the solutions, pressed flat with a cover glass, and examined.

The normal mucous membrane presented the picture described by Bensley and Harvey. There was no acid in the glands, but an abundance in the foveola (blue in cyanamin solution, red in neutral-red solution). There was no acid in the foveola in the hemorrhagic area, or in that immediately surrounding this area. The gland cells in the hemorrhagic area became acid more rapidly than the adjacent more normal cells. There was no evidence of secretion in the parietal cells.

June 30.—Cultures from blood, bile, liver, and joint fluid, were negative, while those from the ulcer showed 50 colonies of streptococci.

The results of this study indicate that the infection inhibits locally the secretory function of the gastric cells, and that the digestion of the damaged cells is due to the gastric juice formed in other portions of the stomach. The infection in the fundus is not essentially different from infection in the duodenum or in the pylorus or in other tissues.

#### THE STREPTOCOCCI

The streptococci isolated from the ulcers produced small, moist, nonadherent, discrete, grayish-brown or grayish-green colonies on blood (human) agar plates, and produced short chains and masses of coccus-like forms (Fig. 1), a diffuse turbidity with a flocculent sediment, and much acid in dextrose broth and ascites dextrose broth. They acidify, but usually do not coagulate, milk. They are free from capsule, are bile-insoluble and freely susceptible to phagocytosis. They do not ferment inulin and produce much acid and precipitate ascites dextrose agar. They resembled very closely those isolated in appendicitis and cholecystitis. In only one instance were there isolated

typical hemolytic streptococci from the ulcer wall, and in no instance from the lymph glands draining the ulcer.

In the primary cultures, smears from single colonies, especially from those in ascites dextrose agar, showed at times so little chain-formation and so many clumps made up of small cocci and indistinct diplococci that it was difficult to decide whether a given colony represented a mixture of streptococci and staphylococci, or a peculiar streptococcus. Subcultures on blood-agar plates or in broth usually cleared up the point.

The cultures from the supposed atrium of infection showed chiefly nonhemolyzing streptococci. In most instances, however, hemolyzing streptococci in small numbers, together with a few colonies of staphylococci, were also present, and the usual number of gram-negative cocci resembling *Micrococcus catarrhalis*. Injection of mixtures of hemolyzing and nonhemolyzing streptococci was usually followed by ulcer and arthritis. The former, in some instances, proved to be due to *Streptococcus viridans*, the latter to hemolytic streptococci. In no instance did the hemolyzing streptococcus show predilection for the stomach.

Streptococci from the most chronic ulcers produced the smallest colonies, the least amount of green on blood-agar plates, and the shortest chains in ascites dextrose broth and other liquid media. Most of the strains resembled *Streptococcus faecalis* (Horder). A number produced a narrow zone of hemolysis peripheral to the primary green zone. Blood corpuscles in broth cultures were usually not dissolved, or were dissolved very slowly. The strains from the two acute ulcers, as did most of the strains from the supposed atrium of infection (Fig. 2) which had affinity for the stomach, produced a larger amount of green on blood agar. With all strains tested, animal passage tended to increase the size of the colonies, and the amount of green-production. Two of the strains (from Cases 773 and 779) appeared to take on hemolytic properties in dogs during, respectively, the 5th and 6th animal passages. The changes which these streptococci underwent are in accord with those observed by me in a special study of this question several years ago.<sup>43</sup>

The property on which the characteristic localization depended, could be preserved best in the depth of the original cultures in ascites dextrose broth and in salt solution, containing pieces of tissue, kept in the ice chest. Cultural and other changes were often not demon-

strable as the strains lost their power to produce ulcer as a result of cultivation on artificial media.

The virulence on isolation was relatively low, as shown by the fact that intraperitoneal injection into mice of large doses of 7 strains isolated from chronic ulcer was followed by recovery in all; as shown also by the fact that 57% of the animals injected were chloroformed after they had seemingly recovered from the effects of even large doses of the culture, and that the blood in 55% was sterile at autopsy, notwithstanding the fact that many animals were examined in from 24 to 48 hours after injection. This is in sharp contrast with the results in the animals showing ulcer following injection of streptococci from sources other than ulcer. Here 91% died from the effects of the injection, and 80% showed streptococci in the blood. The limited power of the strains from ulcer to invade the blood or other structures is shown further by the fact that the cultures from the joint fluid yielded streptococci in only 22% of 40 tested, while the joint fluid in animals showing ulcer after injection with streptococci from sources other than ulcer, showed streptococci in 80% of 39 tested. The injected streptococcus was found in the bile, often in large numbers, in approximately 50% of both sets of animals, irrespective of whether or not there were lesions in the gallbladder or bile ducts. The size and moistness of the colonies, and the amount of green-production on blood agar, became greater as virulence was increased, after successive animal passages. In a number of these strains there appeared a distinct but narrow capsule. The strains from ulcer resembled in virulence and in other features those isolated by Dudgeon and Sargent<sup>38</sup> from the edges of perforating ulcer.

The details of the fermentative powers are reserved for a separate paper. It should be stated, however, that the fermentative powers of 12 strains in the various sugars, were tested repeatedly. The fermentative powers of the various strains were not identical and fluctuated considerably. Acid was produced in dextrose by all. Lactose was fermented in all but 6 instances. Saccharose was fermented in 14 of 20 tests. Acid was produced in raffinose in only 8 of 52 instances, in mannite in 34 of 60 tests, in salicin in 46 of 55 tests, and in inulin in only 3 of 64. The three strains which fermented inulin, produced green colonies on blood agar, and resembled pneumococci morphologically.

Altho the different strains from the ulcers and foci of infection are much alike, their fermentative and other features differ in certain

important respects. They are not sufficiently alike to warrant considering them a distinct or specific species.

#### GENERAL DISCUSSION

The fact that the dogs fed with mixtures of meat, sharp particles of bone, and streptococci, failed to develop ulcer, and the fact that certain persons have swallowed splinters of glass many times without developing ulcer, indicate that local injury by swallowed food, or even local invasion of bacteria from the mucous membrane is rarely, if ever, the cause of ulcer.

In the light of these experiments, the thrombosis in ulcer in man, first observed by Virchow<sup>47</sup> in 1853, and frequently since, must be considered retrograde and secondary to an antecedent localized infection. While it is of importance in preventing the healing of an ulcer already formed, it cannot by itself be the primary cause, because ulcer does not regularly follow obstruction of numerous small arteries,<sup>32, 48, 49, 50, 51</sup> not even after ligating one-third of the arteries to the stomach.<sup>52</sup>

The results of Turck<sup>29</sup> on the production of ulcer in animals by injection and feeding of colon bacilli, possibly applicable in some ulcers, in the light of these findings have little bearing on the chief problem of ulcer of the stomach and of the duodenum in man. Colon bacilli are rarely found in ulcer in man during life, and, if Turck's feeding experiments have a bearing, ulcer of the stomach should occur chiefly in persons with profound malnutrition, the result of improper and insufficient food and unsanitary surroundings.

Since streptococci from certain foci of infection in patients with ulcer tend to produce ulcer of the stomach in animals, might not the frequency of ulcer in the male sex, in certain localities, and during the winter months,<sup>53</sup> be best explained on the basis of a high incidence of throat and other infections? Such infections would afford opportunity for streptococci to acquire affinity for the stomach and to gain entrance into the blood stream.

The ulcers produced in my experiments, just as do spontaneous ulcers in man, tended to heal in the fundus, and to become chronic in the pyloric portion, the lesser curvature, or the duodenum. While the elective affinity of the bacteria for the gastric mucous membrane is the primary cause of the ulceration, certain contributing factors play a definite rôle in making for the chronicity of the ulcer.

The digestive action of the gastric juice has been repeatedly put forth as a cause of ulcer and as the chief factor in preventing the heal-

ing of ulcer. But this is improbable, inasmuch as recent roentgenologic studies<sup>54, 55, 56</sup> have shown that hyperacidity and violent spasms may be present over a period of years from causes outside the stomach without the development of ulcer. Ulceration does not occur along the segment of the stomach thrown into violent spasm directly opposite a chronic ulcer. An example of this is shown in Fig. 24, for which I am indebted to Dr. Carman. The promptness with which defects in the mucous membrane of the stomach heal after excision, after injection of corrosive chemicals, after interference with the blood supply, and after operations, shows how unimportant is the action of the gastric juice. The fact that ulcer occurs in achylia gastrica<sup>57</sup> also supports this view.

Some ulcers in man may be made to heal when the acidity is reduced by the administration of alkalies, as advocated especially by Sippy,<sup>60</sup> or by the alkaline contents of the duodenum, following gastroenterostomy.\* Might not the good effect be due partly to an alkalization of the tissues throughout the body, rather than wholly to local action? The direct digestive action of the hyperacid gastric juice on the floor of the ulcer is believed to prevent the healing. If this is true, chronic ulcer should be found where this action proceeds for the longest time and is most direct; that is, in the acid-secreting portion of the stomach. This is not the case. No matter how much prolonged or increased the action of the gastric juice, the fact remains that its corrosive action must be less in the duodenum than in the stomach, and probably is appreciably less in the pyloric segment and in the lesser curvature, the common sites of both experimental and spontaneous chronic ulcers. Something with greater penetrating power than the gastric juice must first damage the cells before they can be digested.

Clinical, roentgenologic,<sup>54</sup> and experimental studies<sup>3, 5, 58, 59</sup> on the physiology of the stomach prove conclusively that ulcers along the lesser curvature, in the pylorus, and in the duodenum, are especially prone to be associated with abnormal motility of the stomach and spasm of the pylorus, resulting in delayed emptying, hypersecretion, and hyperacidity. This peristaltic unrest produces mechanical injury, necessarily greatest in the relatively fixed points where chronic ulcer occurs,<sup>62</sup> prevents physiologic rest, and hence serves to maintain the primary infection in the margin of the ulcer, at the same time increas-

\* It should be remembered, however, that freedom from symptoms, and even absence of occult blood in the stool, are no proof that a chronic ulcer has healed; for, as Mayo states,<sup>61</sup> "When supposedly cured cases are operated on during the quiescent interval, the ulcer is not found to be cicatrized, but unhealed. Roentgenograms show the same thing."

ing the liability to secondary infection. This mild, but long-continued, traumatism appears to be of greater importance in preventing healing than the direct corrosive action. Infection and infiltration of connective tissue are favored, resulting ultimately in the calloused crater-like ulcer, which for mechanical reasons cannot heal, even tho the infection is reduced to a minimum or is completely overcome.

This conception is in accord with the results obtained by Bolton,<sup>5</sup> who showed that partial closure of the pylorus delayed the healing of ulcers produced by injections of gastrototoxic serum, but only of those that became "septic." It is in accord with Bolton's<sup>63</sup> more recent results in which he again showed that delayed healing of ulcer occurred chiefly in those animals in which the obstruction at the pylorus was so marked as commonly to cause death, and in those which were given abnormal concentrations of hydrochloric acid. It is not at variance with the results of Hamburger and Friedman,<sup>3</sup> who showed that partial obstruction of the pylorus resulting in extreme hypermotility and dilation, delayed the healing of ulcers produced by local injection of silver nitrate, particularly in the pyloric portion. It is in accord with the results of Durante,<sup>23</sup> who produced ulcers by ligating the splanchnic nerves. The ulcers shown by him to be chronic, present evidence of infection. If Durante had searched for bacteria, he would undoubtedly have found them, because in one of the "chronic" ulcers, I demonstrated (after the publication of his paper) not less than 50 cocci and diplococci in the depths of the tissue, which showed leukocytic infiltration; moreover no bacteria could be found in the healing ulcer from the same stomach, which showed no leukocytic infiltration.

Might not this conception best explain the etiologic relationship to ulcer of the vagotonic or neurotic state in general, as emphasized especially by Westphal and Katsch,<sup>64</sup> de Kock,<sup>65</sup> Gunderman,<sup>66</sup> and Eppinger and Hess?<sup>67</sup> Disturbed motility and spasm of the stomach and hyperacidity, occur commonly in neurotic persons.

Moreover, if lesions of the autonomic nervous system are ever a cause of ulcer, as emphasized by Durante's experiments, then it may be suggested in the light of the work by Oftedal and myself on herpes zoster,<sup>68</sup> and other more recent experiments, that streptococci or other bacteria or their toxic products may be the cause of the lesions in the autonomic nervous system.

In support of the view that ulcer of the stomach in the adult is due to streptococci, it should be stated that Gerdine and Helmholz<sup>69</sup> by the use of the same methods have not only shown that a recent epidemic

in Chicago of duodenal ulcer in infants was due to streptococci, but on re-studying the sections of the ulcers from a similar epidemic in Berlin, reported by Helmholtz<sup>70</sup> 7 years ago, they have demonstrated streptococci in all but 4 of 14 ulcers available.\* Furthermore, in a study of the etiology of spontaneous ulcer of the stomach in dogs, calves, cattle, and sheep, in conjunction with Dr. Dart and Dr. Henderson (as yet unpublished), it appears that ulcer in these animals also is due commonly to a circumscribed streptococcal infection.

The occurrence of acute ulcer of the stomach and exacerbations of the symptoms in chronic ulcer in connection with foci of infection; the improvement in symptoms following removal of foci of infection; and the development of new ulcers after excision of ulcer in patients in whom chronic suppurating foci have not been removed—all strongly suggest the etiologic relation between remote foci of infection and ulcer. None of these observations, however, proves the etiology of the ulcer. The demonstration of streptococci in foci of infection in patients with ulcer and in the ulcers themselves, and the fact that they localize in the stomach in animals, furnish what seems to me to be the final proof of the etiology.

The conditions under which streptococci acquire affinity for various organs,<sup>68, 71, 72, 73, 74</sup> are still obscure, but of the existence of this affinity of streptococci in diseases, there is no question. The fact that in some instances streptococci were isolated from relatively insignificant foci of infection, and the fact of their presence in patients with ulcer over a long period suggest, as I have already pointed out,<sup>42</sup> “that differences in the host may afford the peculiar type of reaction or that the individual harbors a particular form of focus of infection, which is favorable for bacteria to acquire the various elective properties.” These observations suggest strongly that while removal of evident foci of infection is important, cure should not always be expected.

The periodic occurrence of exacerbations in symptoms followed by quiescent intervals in chronic ulcer would seem to be best explained on the basis of infection, the former being due to a lighting up of the dormant infection or to re-infection from a focal source when immunity is low and the latter to quiescence of the infection, the result of heightened local or general immunity.

\* One of the strains from a duodenal ulcer in an infant isolated by them, resembled very closely the strains isolated from ulcers in adults by Sanford and myself. This strain localized electively in the stomach of rabbits and dogs, producing ulcer, which proved to be due to local infection after intravenous injections made by Gerdine, by Hardt, and by myself. Re-injection again produced ulcer.

## SUMMARY

The ulcers produced by the injection of streptococci resemble those in man in location, in gross and microscopic appearance, and in that they tend to become chronic, to perforate, and to cause severe or fatal hemorrhage.

Streptococci having a characteristic affinity, for the stomach and the duodenum, have been repeatedly isolated from various foci of infection in patients with ulcer and from the ulcers themselves. They tend to disappear from the circulation and do not commonly produce marked lesions otherwise. They have been isolated from ulcers in animals, and ulcer has again been produced on their re-injection. Filtrates of these cultures show no special tendency to produce ulcer. The necessary requirements have been fulfilled to warrant the conclusion that the usual ulcer of the stomach and of the duodenum in man is primarily due to a localized hematogenous infection of the mucous membrane by streptococci.

## BIBLIOGRAPHY

1. Quinke and Dättwyler: *Cor.-Bl. f. schweiz. Aerzte*, 1875, p. 101. *Deutsch. med. Wchnschr.*, 1882, 6, 79. Quoted by Ewald, *Diseases of the Stomach*, 1902, p. 382.
2. Becker: *Berl. klin. Wchnschr.*, 1887, 24, p. 369. Quoted by Ewald, *Diseases of the Stomach*, 1902, p. 384.
3. Friedman and Hamburger: *Jour. Am. Med. Assn.*, 1914, 63, p. 380.
4. Licini: *Beitr. z. klin. Chir.*, 1912, 79, p. 462.
5. Bolton: *Ulcer of the Stomach*, 1913, p. 59.
6. Cohnheim: *Lectures on General Pathology*, 1890, 3, p. 879.
7. Schridde: *Verhandl. d. deutsch. path. Gesellsch.*, 1907, 11, p. 234. Quoted by Bolton, *l. c.*, p. 78.
8. Rehfuss: *Univ. Penn. Med. Bull.*, 1909, 22, p. 105.
9. Smith: *Jour. Med. Research*, 1914, 30, p. 147.
10. Sellards: *Arch. Int. Med.*, 1909, 4, p. 502. *Abstr. Jour. Am. Med. Assn.*, 1909, 53, p. 24.
11. Elliott: *Quart. Jour. Med.*, 1914, 7, p. 119. *Abstr. Jour. Am. Med. Assn.*, 1914, 62, p. 576.
12. Friedman: *Jour. Med. Research*, 1915, 32, p. 287.
13. Enriquez and Hallion: *Compt. rend. Soc. de biol.*, 1893, 5, p. 1025. Quoted by Bolton, *l. c.*, p. 61.
14. Rosenau and Anderson: *Jour. Infect. Dis.*, 1907, 4, p. 1.
15. Gioffi: Quoted by Pende, *l. c.*
16. Pende: *Fisiopatologia dell' apparte parasimpatico*, 1906.
17. Gibelli: *Pathologica*, 1909, p. 131.
18. Finzi: *Virchow's Arch. f. path. Anat.*, 1913, 214, p. 413.
19. Mann: *Jour. Exper. Med.*, 1916, 23, p. 203.
20. Elliott: *Jour. Physiol.*, 1914, 49, p. 38.
21. Kawamura: *Deutsch. Ztschr. f. Chir.*, 1911, 109, p. 540.
22. Vedova: *Suppl. Policlinico*, 1900, 6, p. 1153.

23. Durante: *Pathologica*, 1912-13, 5, p. 631. *Surg., Gynec. and Obst.*, 1916, 22, p. 399.
24. Ewald: *Klinik der Verdammungskrankheiten*, 1890-93, 1, p. 122. Quoted by Ewald, 1, c., p. 382.
25. Charrin and Ruffer: *Bull. Soc. anat. de Paris*, 1889, 3, p. 18. Quoted by Bolton, 1, c., p. 61.
26. Chantemesse and Vidal: *Bull. de l'Acad. de méd de Paris*, 1888, 14, p. 522. Quoted by Bolton, 1, c., pp. 61, 63.
27. Wurtz and Leudet: *Arch de méd. exper. et d'anat. path.*, 1891, 3, p. 485. Quoted by Bolton, 1, c., p. 94.
28. Rodet and Zaidmann: *Compt. rend. Soc. de biol.*, 1900, 52, p. 1007.
29. Turck: *Brit. Med. Jour.*, 1907, 1, p. 922, and other papers.
30. Steinharter: *Lancet-Clinic*, 1914, 111, p. 88. *Boston Med. and Surg. Jour.*, 1913, 169, p. 81.
31. Lebert: *Traité d. anat. path.*, 1857, 1, p. 537. Quoted by Bolton, 1, c., p. 61.
32. Cohn: *Klinik der embolischen Gefässkrankheiten*, 1860, p. 523.
33. Letulle: *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1888, 5, p. 360. Quoted by Bolton, 1, c., p. 18.
34. Bezançon and Griffon: *Bull. et mém. Soc. anat. de Paris*, 1899, 1, p. 409. Quoted by Bolton, 1, c., p. 62.
35. Dieulafoy: *Textbook of Medicine*, 1902, p. 679.
36. Ewald: *Diseases of the Stomach*, 1902, pp. 183, 391.
37. Boettcher: *Dorparter Med. Ztschr.*, 1874, 5, p. 148. Quoted by Bolton, 1, c., p. 60.
38. Dudgeon and Sargent: *The Bacteriology of Peritonitis*, 1905, p. 58.
39. Hutrya and Marek: *Spezielle Pathologie und Therapie der Haustiere*, 1913, 2, p. 331.
40. Rosenow: *Jour. Am. Med. Assn.*, 1913, 61, p. 1847.
41. Rosenow and Sanford: *Jour. Infect. Dis.*, 1915, 17, p. 219.
42. Rosenow: *Jour. Am. Med. Assn.*, 1915, 65, p. 1687.
43. Rosenow: *Jour. Infect. Dis.*, 1914, 14, p. 1.
44. Mayo: *Boston Med. and Surg. Jour.*, 1914, 170, p. 149.
45. Von Korczynski and Jaworski: *Deutsch. Arch. f. klin. Med.*, 1891, 47, p. 586. Quoted by Ewald, 1, c., p. 194.
46. Bensley and Harvey: *Tr. Chicago Path. Soc.*, 1913, 9, p. 14.
47. Virchow: *Virchow's Arch. f. path. Anat.*, 1853, 5, p. 362. Quoted by Bolton, 1, c., p. 396.
48. Möller: *Ergebn. d. inn. Med. u. Kinderh.*, 1911, 7, p. 520.
49. Pavy: *Guy's Hosp. Rep.*, 1867, 13, p. 494. *Roy. Soc. Proc.*, 1863, p. 386. Quoted by Bolton, 1, c., p. 70.
50. Roth: *Virchow's Arch. f. path. Anat.*, 1869, 45, p. 300.
51. Litten: *Virchow's Arch. f. path. Anat.*, 1875, 63, p. 289. Quoted by Trotter, *Embolism and Thrombosis*, 1913.
52. Lithauer: *Virchow's Arch. f. path. Anat.*, 1909, 195, p. 317. Quoted by Bolton, 1, c., p. 128.
53. Moynihan: *Duodenal Ulcer*, 1910, pp. 106, 251.
54. Carman: *Am. Jour. Roentgenol.*, 1914, 1, p. 233.
55. Rieder: *Verhandl. d. deutsch. Kong. f. inn. Med.*, 1912, 29, p. 17.
56. Holzknecht and Luger: *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1913, 26, p. 669.
57. Eusterman: *Jour. Am. Med. Assn.*, 1915, 65, p. 1500.
58. Cannon: *The Mechanical Factors of Digestion*, 1911.

59. Carlson: New York Med. Jour., 1915, 102, p. 1004. Also numerous recent publications in the Am. Jour. Physiol.
60. Sippy: Jour. Am. Med. Assn., 1915, 64, p. 1625.
61. Mayo: Jour. Am. Med. Assn., 1915, 65, p. 1069.
62. Stromeyer: Beitr. z. path. Anat. u. z. allg. Path., 1912, 54, p. 1.
63. Bolton: Jour. Path. and Bacteriol., 1915, 20, p. 133.
64. Westphal and Katsch: Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1913, 26, p. 391.
65. De Kock: Nederl. Tijdschr. v. Geneesk, 1913, 2, p. 449.
66. Gundermann: Beitr. z. klin. Chir., 1914, 90, p. 1.
67. Eppinger and Hess: Sammlung klinischer Abhandlungen über Pathologie und Therapie der Stoffwechsel und Ernährungsstörungen, 1910, Vols. 9 and 10.
68. Rosenow and Oftedal: Jour. Am. Med. Assn., 1915, 64, p. 1968.
69. Gerdine and Helmholz: Am. Jour. Dis. Child., 1915, 10, p. 397.
70. Helmholz: Arch. Pediat., 1909, 26, p. 661.
71. Rosenow: Jour. Infect. Dis., 1915, 16, p. 240.
72. Rosenow: Jour. Am. Med. Assn., 1914, 63, p. 1835.
73. Rosenow: Jour. Infect. Dis., 1915, 16, p. 367.
74. Irons, Brown, and Nadler: Jour. Infect. Dis., 1916, 18, p. 315.

## EXPLANATION OF PLATES

## PLATE 5

FIG. 1. Smear from a 24-hour ascites-dextrose-broth culture of the streptococcus isolated from a duodenal ulcer in man (Case 773). Gram-Weigert stain.  $\times 1,200$ .

FIG. 2. Smear from a 24-hour ascites-dextrose-broth culture of the streptococcus isolated from an infected tooth in a patient with ulcer of the stomach. The smear was made at the time that the strain was proved to have affinity for the stomach in animals (Case 52). Gram stain.  $\times 1,200$ .

## PLATE 6

FIG. 3. Multiple hemorrhages, necroses, and ulcerations of the mucous membrane of the stomach of Dog 42, Case 779, 4 days after an intravenous injection of a streptococcus isolated from a gastric ulcer in man and passed through one animal. Natural size.

FIG. 4. Section through the area of the stomach at "a" in Fig. 3, showing the sloughing mucous membrane, the marked hemorrhage, leukocytic infiltration, the poorly staining cells, and the aggregations of leukocytes in the dilated blood vessels in the submucosa. Hematoxylin and eosin.  $\times 50$ .

## PLATE 7

FIG. 5. A portion of the infiltrated and necrotic mucous membrane of the ulcer shown in Fig. 4, under higher magnification. Marked disintegration and disappearance of the chief cells, leukocytic infiltration, and relatively normal appearance of the parietal cells. Hematoxylin and eosin.  $\times 400$ .

FIG. 6. Photomicrograph of a mass of diplococci and streptococci in the sloughing portion of the ulcer shown in Fig. 4. Gram-Weigert stain.  $\times 1,000$ .

## PLATE 8

FIG. 7. Chronic ulcer of the duodenum in Dog 37, Case 773, 14 weeks after intravenous injection of a streptococcus from an excised ulcer of the duodenum in man. Natural size.

FIG. 8. Section of the ulcer shown in Fig. 7. Invasion of the muscular coat by connective tissue at "a" and peritoneal adhesions at "b." Hematoxylin and eosin.  $\times 80$ .

FIG. 9. Diplococci in the margin of the ulcer shown in Fig. 8.  $\times 1,000$ .

## PLATE 9

FIG. 10. Ulcer of duodenum at "a" in Dog 156 (Case 112) 18 days after intravenous injection of a streptococcus from the peritoneal coat of an ulcer in the duodenum in man (Case 112). Natural size.

FIG. 11. Section of the ulcer in the duodenum shown in Fig. 10. Ragged base, poorly staining connective-tissue stroma, moderate leukocytic infiltration, thrombosed vessels (b), and a large number of leukocytes in the thrombi. Hematoxylin and eosin.  $\times 120$ .

FIG. 12. Photomicrograph of two diplococci found in the thrombosed vessel at "a" in the ulcer shown in Fig. 11. Gram-Weigert stain.  $\times 1,000$ .

## PLATE 10

FIG. 13. Streptococci in the peritoneal coat of a perforating ulcer of the stomach in a rabbit (R652) 35 days after intravenous injection of the streptococcus isolated from a lymph-gland draining a perforating ulcer of the duodenum in man (Case 947). Gram-Weigert stain.  $\times 1,200$ .

FIG. 14. Marked ulceration of the stomach in a guinea-pig (P12) 24 hours after intravenous injection of a streptococcus from a suppurating frontal sinus in a man with ulcer of the stomach (Case 213).

FIG. 15. Chains of diplococci in the margin of the ulcerated mucous membrane shown in Fig. 14. Gram-Weigert stain.  $\times 1,000$ .

## PLATE 11

FIG. 16. Hemorrhage in the duodenum in a rabbit (R792) 48 hours after intravenous injection of a streptococcus from the tonsil in a patient with arthritis deformans and probable ulcer of the stomach (Case 163). Natural size.

FIG. 17. Ulcer of the mucous membrane of the stomach in a rabbit (R787) 3 days after intravenous injection of an emulsion of the tonsils from a case of arthritis deformans with symptoms suggesting ulcer of the stomach (Case 163). Note the dark radiating areas at "b" and the apex of the ulcerated area shown at "a." Hematoxylin and eosin.  $\times 60$ .

FIG. 18. A higher magnification of the dark radiating area at "b" in Fig. 17, showing an enormous number of streptococci.  $\times 1,200$ .

## PLATE 12

FIG. 19. Section of the wall of the stomach in a rabbit (R68) showing wedge-shaped area of leukocytic infiltration, hemorrhage, and beginning ulceration, 48 hours after intravenous injection of a streptococcus isolated from the tonsil in herpes zoster and passed through one animal. Hematoxylin and eosin. (Case 281.)  $\times 80$ .

FIG. 20. Streptococci at the apex of the wedge-shaped area shown in Fig. 19. Gram-Weigert.  $\times 1,200$ .

## PLATE 13

FIG. 21. Section of an ulcer of the stomach in a dog (D22) 12 days after intravenous injection of a streptococcus from rheumatism. Note its wedge shape and the round-cell infiltration at "a" between the necrotic and more normal tissue. Hematoxylin and eosin.  $\times 60$ .

FIG. 22. Section through the base of a large ulcer in the duodenum in Dog 25, Case 531, 19 days after intravenous injection of a streptococcus isolated 11 years previously as a pneumococcus. Marked hemorrhage, leukocytic infiltration, and connective-tissue formation. Neutral gentian.  $\times 60$ .

FIG. 23. "Follicular" ulcer of the pylorus of a dog following repeated injections of a streptococcus from a lymph gland at the pole of the thyroid in exophthalmic goiter. There is marked hemorrhage in the submucosa and surrounding the lymph follicle, infiltration, necrosis, connective-tissue formation; the muscular layer is normal. Neutral gentian.  $\times 40$ .

## PLATE 14

FIG. 24. Woman aged 52 years. Small bulb-like projection from lesser curvature in pars media, at "A," which is the crater of a penetrating ulcer. At "B" is shown the cramp-like constriction (incisura) of the circular muscle fibers. Operative findings: Gastric ulcer high on the lesser curvature. Moderate hour-glass contraction (Carman—Case A—107649).

PLATE 5

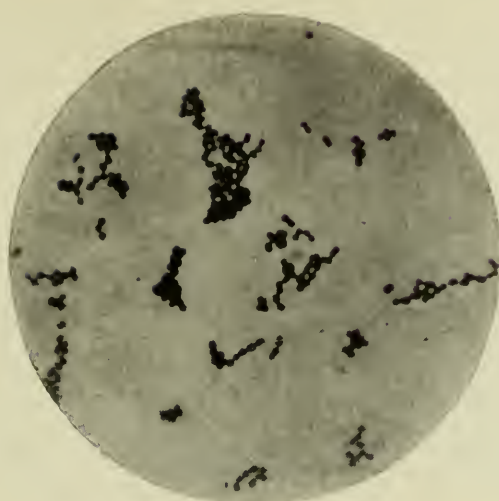


Figure 1

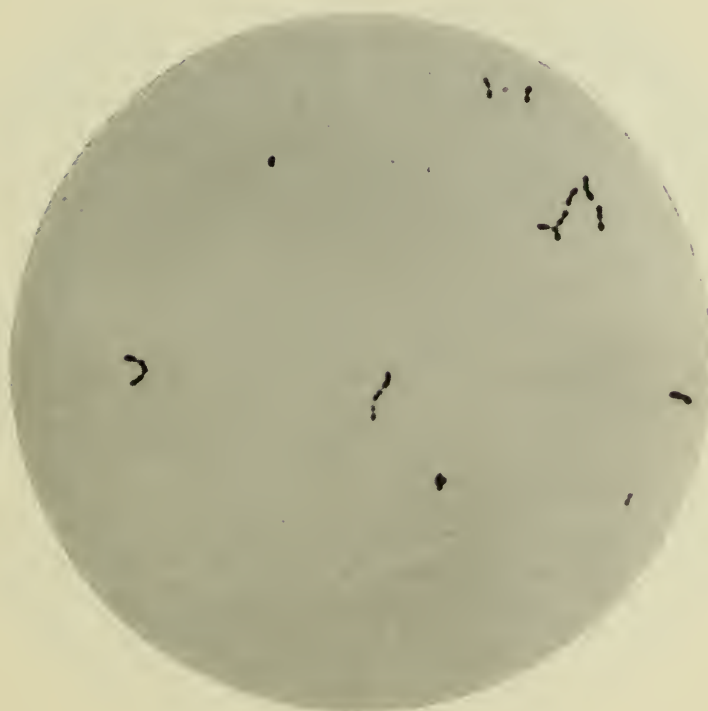


Figure 2



PLATE 6

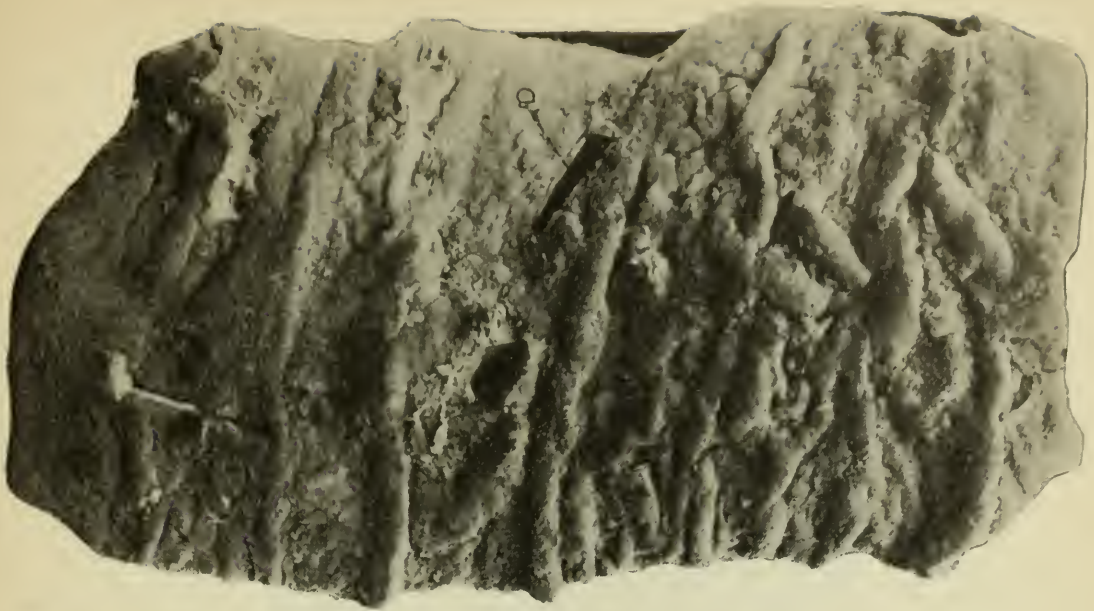


Figure 3

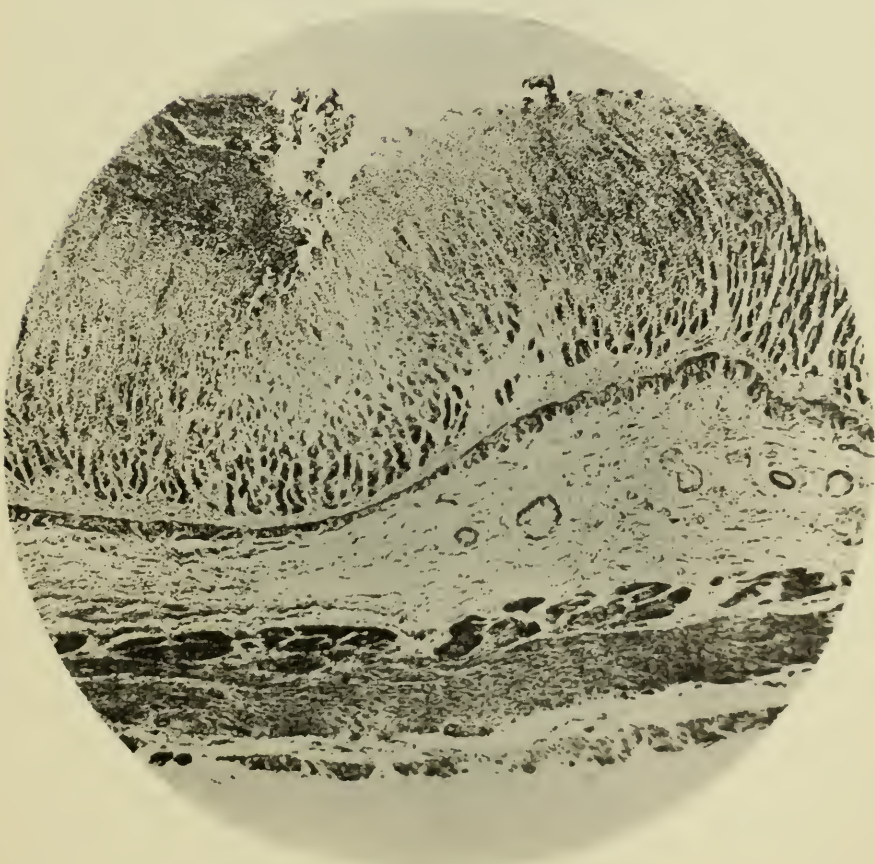


Figure 4



PLATE 7

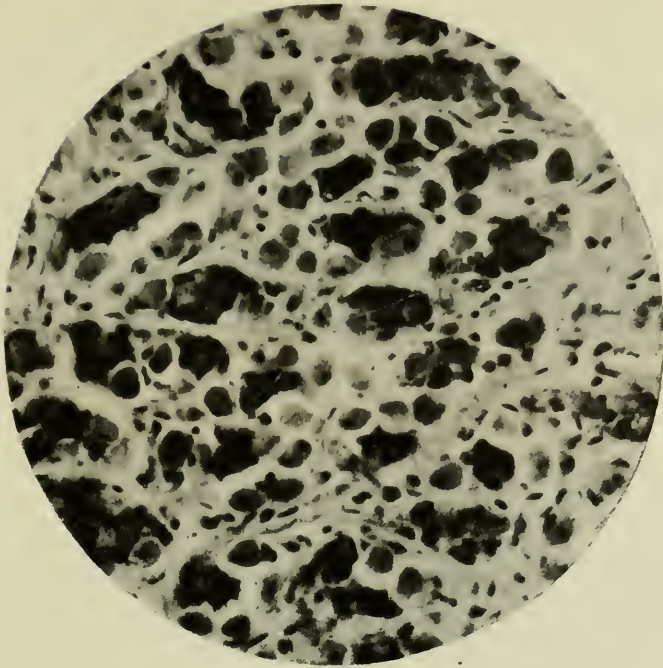


Figure 5



Figure 6



PLATE 8



Figure 7



Figure 8

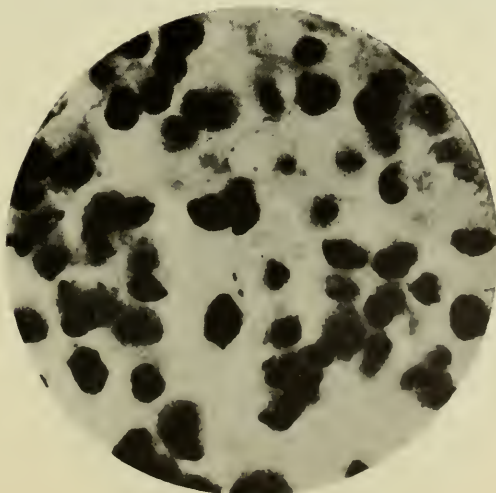


Figure 9





Figure 10

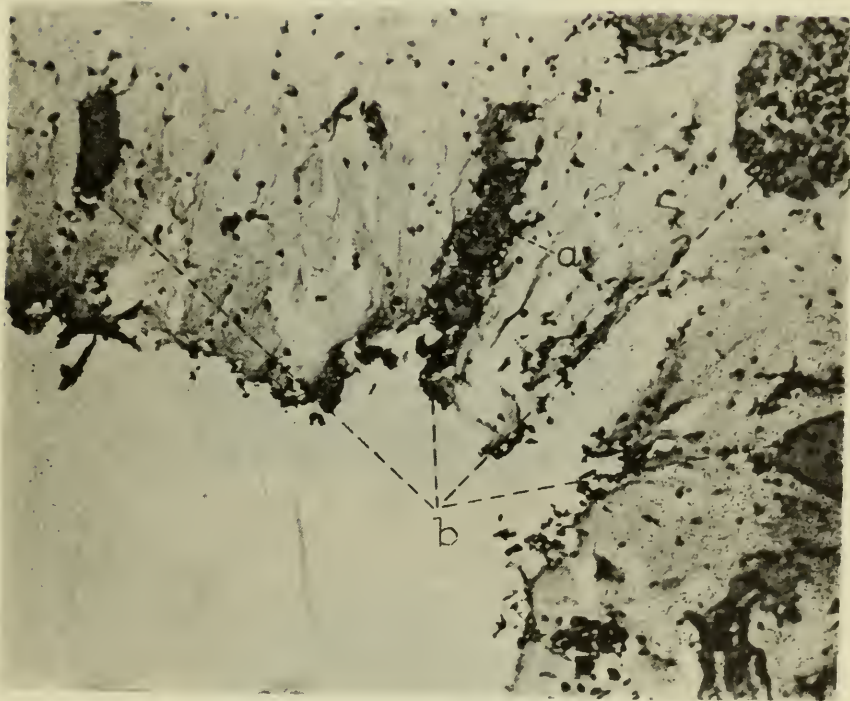


Figure 11

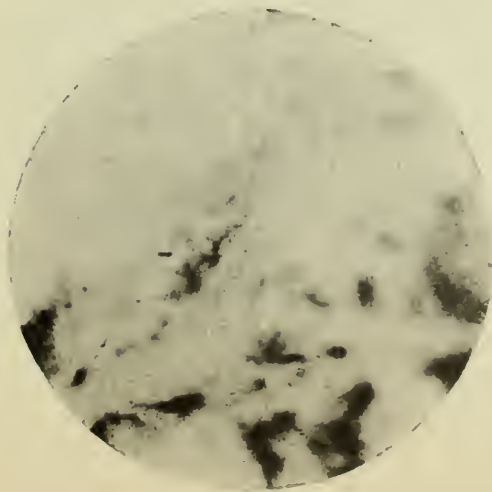


Figure 12



PLATE 10

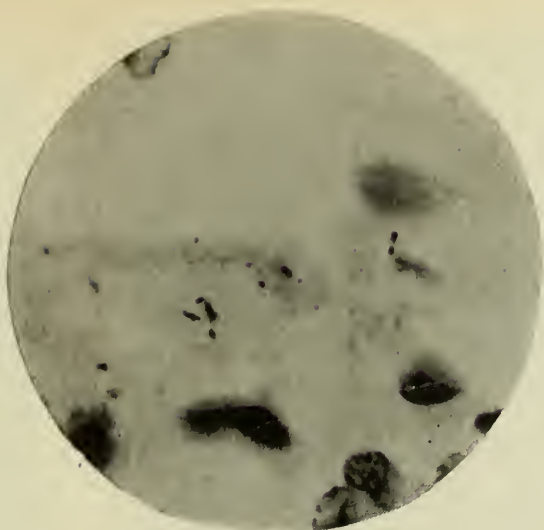


Figure 13

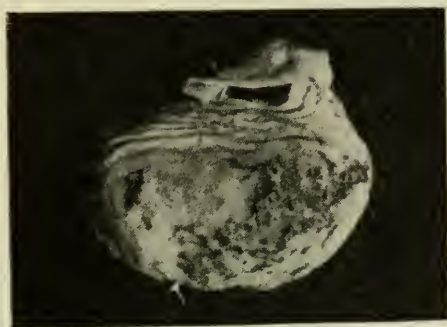


Figure 14

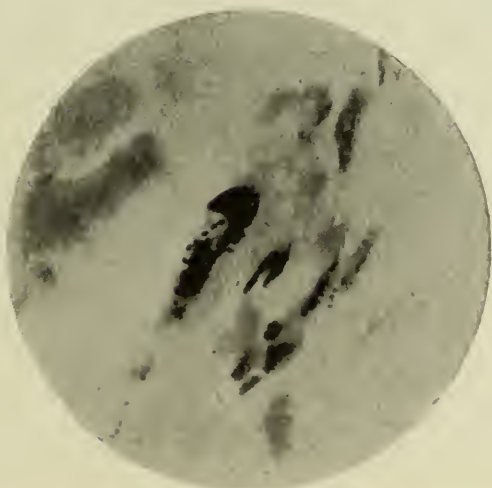


Figure 15



PLATE 11



Figure 16



Figure 17

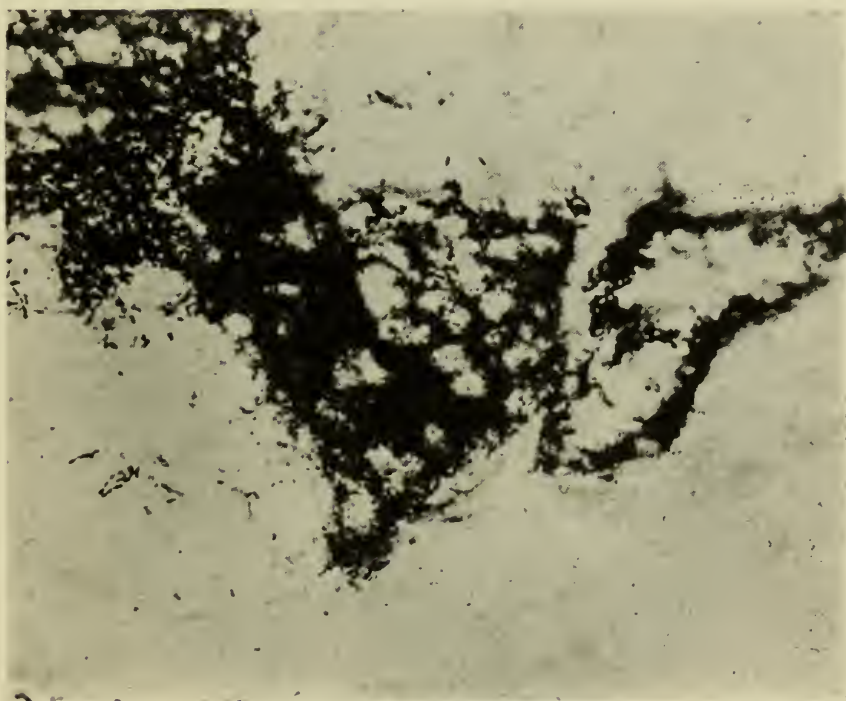


Figure 18



PLATE 12

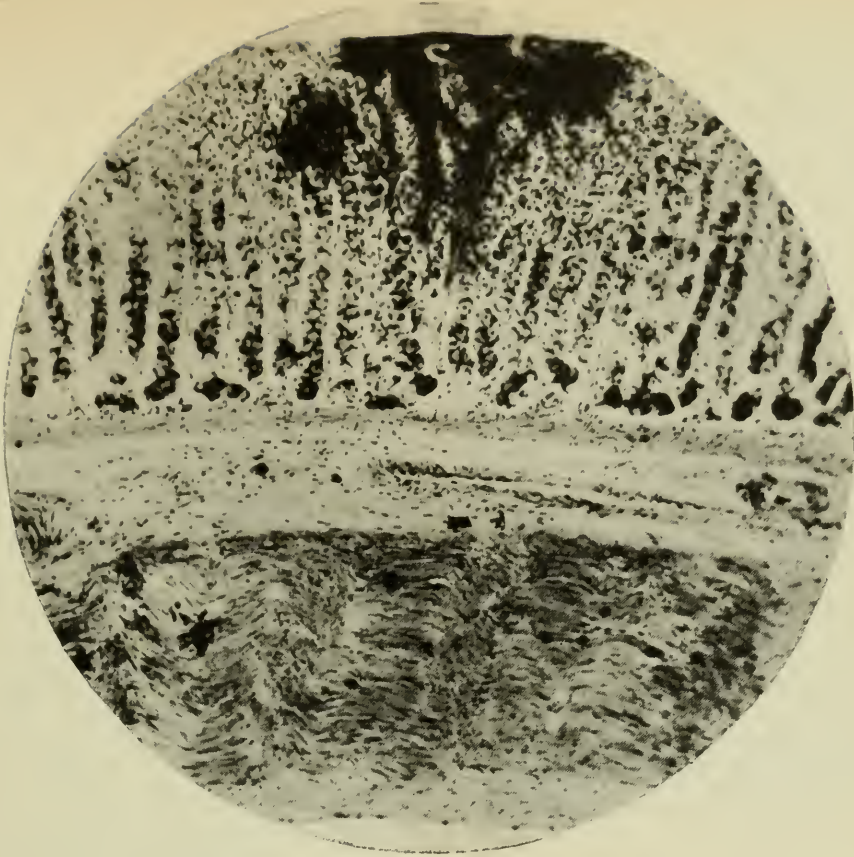


Figure 19

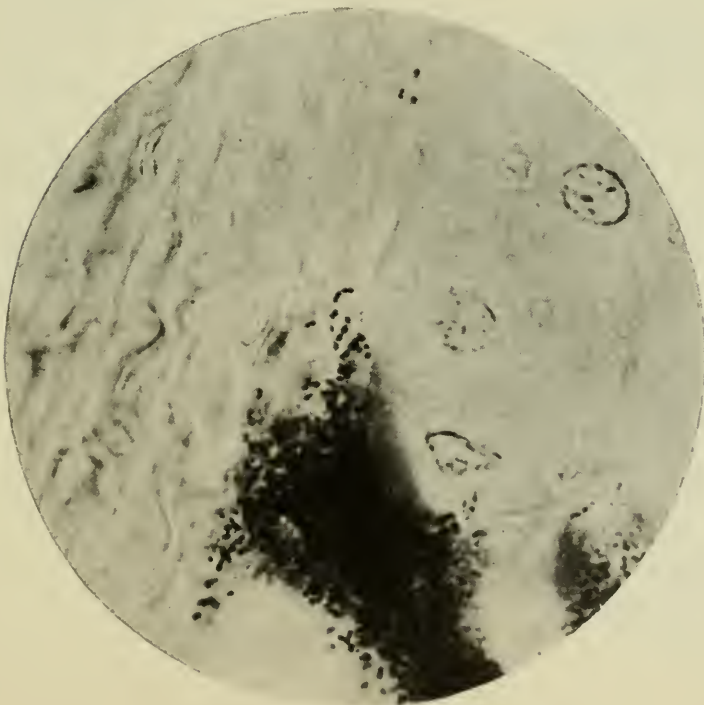


Figure 20



PLATE 13

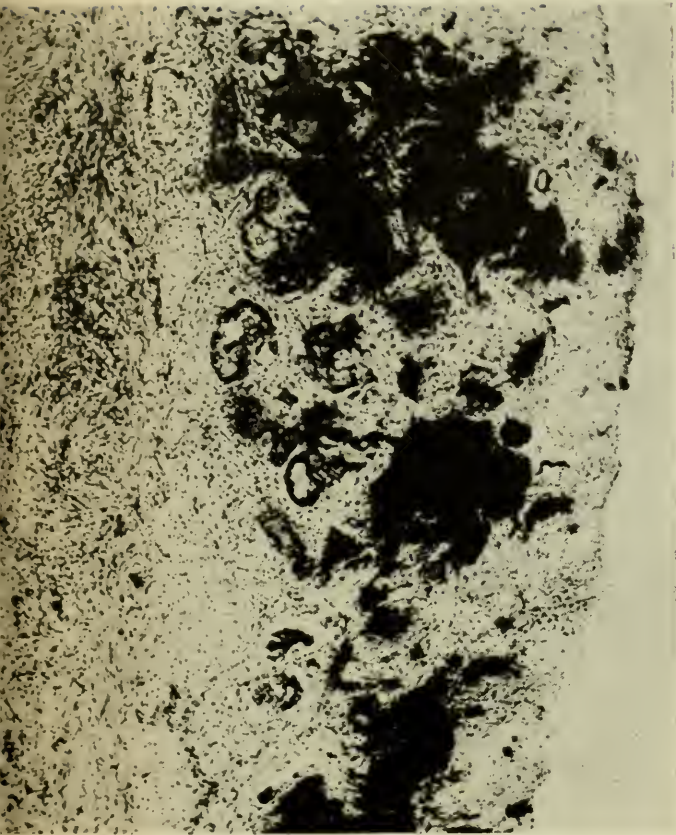


Figure 22



Figure 21

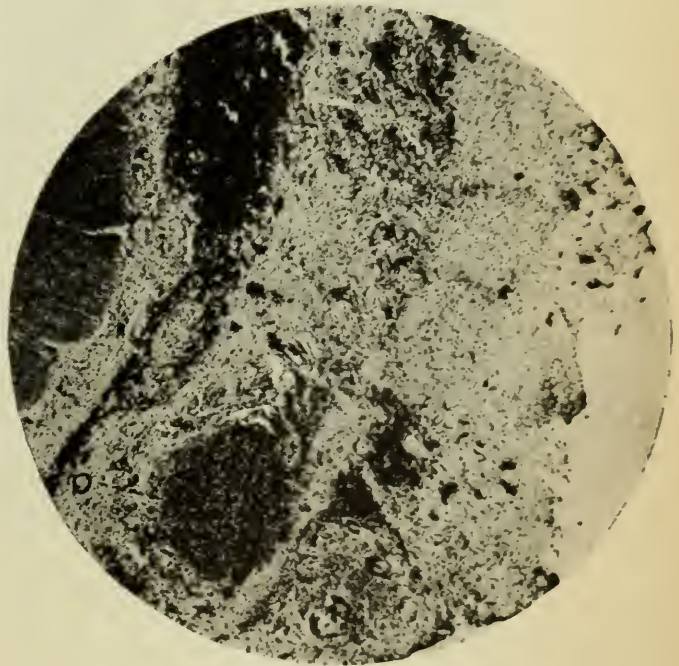


Figure 23



PLATE 14

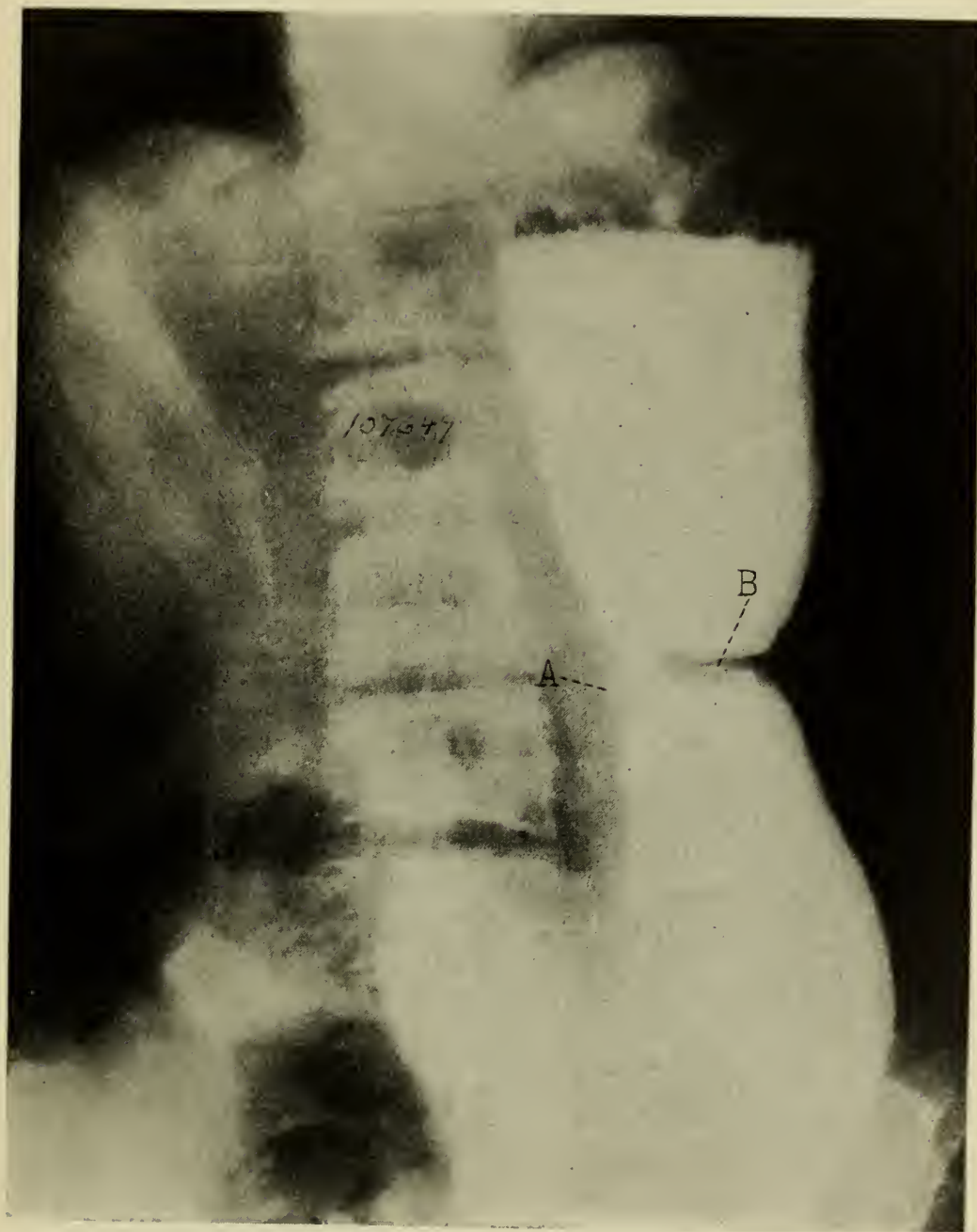


Figure 24



# STUDIES ON FORAGE POISONING \*

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## I

### PATHOLOGIC CHANGES IN A DISEASE IN HORSES RESEMBLING FORAGE POISONING

Previous histo-pathological observations on a disease in horses resembling forage poisoning have been largely confined to the central nervous system.

MacCullum<sup>1</sup> found a softened condition of the anterior region of the brain in gross examination, and in microscopical preparations a complete destruction of the brain substance with colloid degeneration. Congestion was marked, and there were extravasations of the cellular elements of the blood as well as hyaline degeneration of the vessel wall. Moore<sup>2</sup> observed no marked changes in the encephalon. McCarthy and Ravenel<sup>3</sup> described a small round-cell infiltration of the Gasserian ganglia, degenerative changes in the laryngeal nerves, and congestion and capillary hemorrhage of the cerebral and cerebellar cortex. Gross lesions were also observed in the enteron membrane, particularly in the anterior gastro-intestinal tract. Udall<sup>4</sup> in histologic preparations from the hippocampus and the cerebral lobes of the brain found no changes. In more extensive histological examinations in other cases he discovered changes in the olfactory tract and bulb, as well as vascular derangements consisting of lymphocytic infiltrations. Joest and Degen<sup>5</sup> called attention to cell inclusions in the ganglion cells of Ammon's horn when stained with a modified Negri body stain. Mohler<sup>6</sup> noted perivascular round-cell infiltration in the olfactory lobe and the hippocampus. Milks,<sup>7</sup> in 5 cases, noted few changes in the nervous system other than congestion in the meninges. The kidneys, however, disclosed hyperemia and a slight degeneration and desquamation of the tubular epithelium. The liver showed passive congestion. The intestines presented hemorrhages in the mucosa with a thickening of the mucous and submucous layers. No changes were found in the spleen other than a possible increase in white corpuscles. Milks qualified the significance of these changes in view of possible postmortem changes due to climatic conditions.

\* Received for publication March 20, 1916.

<sup>1</sup> Jour. Exper. Med., 1899, 4, p. 409.

<sup>2</sup> Pathology of Infectious Diseases of Animals, 1908, p. 515.

<sup>3</sup> Jour. Med. Research, 1903, 1, p. 243.

<sup>4</sup> Cornell Veterinarian, 1913, p. 17.

<sup>5</sup> Cited by Hutya and Marek, Pathology and Therapeutics of the Diseases of Domestic Animals, 1912, 2, p. 613.

<sup>6</sup> U. S. Bur. An. Ind., No. 65, 1914.

<sup>7</sup> Bull. Louisiana Agr. Exper. Sta., No. 106, 1908.

With few exceptions the authors consulted have confined their observations to the central nervous system, because the clinical symptoms suggest that the primary focus of the affection is in the central nervous system. A summary of the principal changes described in the literature shows that uniform changes have not been observed. A congested condition of the brain and its appendages has been frequently noted, but no one gross or histologic alteration described can be considered pathognomonic.

In our work the tissues studied were secured from 7 horses fatally affected as the consequence of feeding on an oat hay.<sup>8</sup> The animals were killed when in a moribund condition, and the organs removed to the laboratory in less than 1 hour after death. These precautions, as well as weather conditions during the early spring of 1915, preclude the possibility of marked postmortem changes.

Specimens were fixed in formalin and in Zenker's fluid, in the usual manner. Sections were stained in hematoxylin-eosin and osmic acid for fat.

*Brain.*—The brain revealed congestion, the vessels of the various regions of the brain and of the cord being engorged and surrounded by leukocytic and round-cell extravasations. In no region of the cord or brain did we find marked degenerative changes. Cell inclusions, mentioned by Joest and Degen, were not observed.

*Heart.*—Macroscopically the heart contained a few punctate and ecchymotic hemorrhages in the myocardium, and petechial hemorrhages in the mitral and tricuspid valves. The blood was dark and often coagulated. Microscopically, marked congestion and necrosis of the muscle fibers were observed. An infiltration of endothelial cells accompanied the necrosis.

*Lungs.*—The gross appearance of the lungs was that of passive congestion, more intense in some cases than in others, with occasional small defined areas of hypostatic congestion. In one case hemorrhagic suggillations were presented, with a few indistinct punctate subpleural hemorrhages. Microscopically the vessels were engorged, but no marked structural or cellular changes were noted.

*Stomach and Intestinal Tract.*—Gross lesions in the stomach and intestinal tract were not constant, but when observed consisted of small circumscribed areas of hyperemia often accompanied by hemorrhage, a yellowish-white catarrhal exudate adhering to the wall. On the serous surface of the small intestine petechial hemorrhages were sparsely scattered, while in one instance an area of the serous coat of the intestine, approximately 15 cm. in length, was markedly hemorrhagic. In one case the gastric mucosa, and in other cases the intestinal mucosa, was mildly congested in small circumscribed areas. In the cecum and colon the mucosa and submucosa were thickened, and occasionally punctate submucous hemorrhages were seen. The mesenteric lymph nodes appeared slightly swollen and mildly congested in a few cases. Microscopically the mucosa of the stomach showed hemorrhage and exudation, in which poly-

<sup>8</sup> Graham and Himmelberger: Proceedings of the U. S. Live Stock Sanitary Association, 1915. Jour. Am. Vet. Med. Assn., 1916, 48, p. 574.

morphonuclear leukocytes were abundant. The microscopical preparations involving areas of the small and large intestines showed similar changes.

*Liver.*—The liver was in some cases congested. On section small areas of a yellowish color were seen near the margin. Blood often dripped from the cut surface, tho some cases showed no excessive blood content. Histologically the general structure of the liver lobule appeared normal under low magnification, but under higher power, changes of considerable extent were observed. The liver cells were either devoid of nuclei or stained very poorly. At times the degenerative changes were of a fatty nature, the fatty droplets being found within the cytoplasm of the cell and sometimes within the nucleus itself. The cells oftentimes appeared swollen and many lacked nuclei. On the other hand, a superabundance of nuclei appeared to be present in other preparations of this organ, suggesting a reparative process. The capillaries and other vessels were usually engorged, and in the mesh work formed by the capillaries a debris of broken down cells and fragmented nuclei was found. The form of necrosis observed was central. In some of the lobules the necrosis appeared to be on but one side of the central vein and was often complicated by hemorrhage. The blood within the vessels, both central and peripheral, contained large numbers of polymorphonuclear leukocytes.

*Kidneys.*—The kidneys were congested and in some cases a few minute petechial hemorrhages beneath the capsule were observed in the cortex on section. Microscopically a complicated picture was presented. The disturbance of the glomeruli was extensive. The lining of Bowman's capsule showed a distinct desquamation. The capsular space of some contained both red and white blood cells, a degenerative detritus, and fragmented nuclei. The glomerular tuft was swollen and congested, occupying the capsular space, tho in some cases very little of it remained, the space being occupied to a greater or less extent by a cellular debris. The convoluted tubules showed partial destruction of the tubular epithelium. The epithelial cells were devoid of nuclei; in the lumen of the tubule free nuclei and fragments of degenerated cells could be seen. The capillaries were congested and distended, occasionally obliterating the uriniferous tubules. Within the capillaries there was an accumulation of polymorphonuclear and endothelial leukocytes. The most constant microscopic alterations observed in the 7 cases were those of a hemorrhagic nephritis and a marked injection of the vessels, resulting in the obliteration of adjacent structures.

The gross macroscopic lesions mentioned were not observed in the same degree in each animal, but are referred to collectively, as the various organs were apparently normal in some necropsies. The clinical manifestations in affected animals would have led one to expect extensive changes of more moment, especially in the nervous system. Aside from the congestion noted, the central nervous system was apparently not altered in proportion to the nervous manifestations. According to Adami, some of the most difficult conditions to classify are evidently due to and dependent on circulating toxins which may not produce extensive cellular changes.

The changes in the liver are subject to various interpretations. The congestion may have been due to cardiac or other lesions. The

necrosis was of the type considered to be of toxic origin, which includes mineral poisons or bacterial toxins generated within or without the body. Necrotic areas, however, may be found in the liver of apparently healthy animals shortly after death and this fact renders uncertain the exact interpretation of these changes.

The capillary hemorrhage and nephritis may have been the result of any injurious substance exerting an effect on the capillary endothelium. Disturbed heart action and renal function often accompany each other, and in clinical cases a weakened heart action was frequently observed. Cardiac disturbances may also have been partly responsible for the congested condition in the various organs.

The kidney alteration may exercise considerable effect on the bodily health. The lesions observed were not secondary to decumbency, as animals from which tissue was examined were destroyed to avoid such complications. According to von Limbeck,<sup>9</sup> uremia due to disturbed kidney function is the result of retained substances gaining entrance to the circulation.

The gastro-intestinal lesions may have been due to the direct action of poisonous material, or secondary to changes in some other organ, or due to circulating toxins.

While the pathologic presentations in the various tissues examined are not diagnostic, since no pathognomonic importance can be ascribed to them, the changes found collectively are suggestive of a toxemia-like condition in so far as the type of the disease we have studied is concerned.

## II

### A PATHOGENIC BACILLUS ISOLATED FROM OAT HAY

During the winter of 1914-1915 a disease among horses and mules resembling forage poisoning occurred on the Griffith stock farm in central Kentucky; it was later demonstrated by feeding experiments that of the various feeds on the farm the oat hay was primarily responsible. From the report on feeding experiments with this forage the following summary is quoted:

"It is evident from these observations that a particular lot of oat hay was responsible for the outbreak of forage poisoning occurring on the Griffith farm and that this disease could be induced in experimental animals (the horse) by the feeding of this material over a period of time which resembled in a measure the incubation period of some infectious animal diseases; also that drinking water contaminated with these oats resulted fatally when given to experimental animals. Other animal species, i. e., guinea-pigs, rabbits, chickens, swine, white mice,

<sup>9</sup> Arch. f. exper. Path. u. Pharmacol., 1892, 30, p. 180.

goats and sheep, were more resistant and apparently nonsusceptible. It is of interest to note that the oat hay responsible for this disease, tho apparently clean, was later found to be contaminated with the excreta of chickens, which on being fed disguised in wholesome feed resulted fatally. Up to this time, however, we have not been able to locate the causative factor of the disease occurring as a result of feeding this particular lot of oat hay, nor have we been able to show that oat hay from other sources produces forage poisoning in horses and mules."

Much uncertainty exists regarding the cause of forage poisoning; in fact so many etiologic factors have been mentioned that one specific organism cannot be held responsible for all the diseases of horse and mule stock under this caption. In North America moulds of different varieties occurring on vegetation have been incriminated, but feeding experiments with some of the cryptogams isolated from apparently poisonous feed and cultivated under laboratory conditions, suggest only remote connection between some of the moulds referred to in the literature and the disease in question.

Mohler, Mayo, Klimmer, Haslam, and many others observing this disease, consider moulds a possible factor, notwithstanding some of the negative results reported from feeding moulds to experimental horses. A recent bulletin issued by Brown and Ranck of Mississippi<sup>10</sup> describes toxic symptoms and death in guinea-pigs and calves following the injection of the alcoholic extract of the sclerotia of *Claviceps paspali* and the feeding of the heads of wild grass ("paspalum") infected with *Claviceps paspali*, and indicates that this fungus possesses toxic properties. It is possible that certain fungi developing in their natural habitat produce poisonous substances which cannot be reproduced under laboratory conditions, but the moulds commonly found on maize and other forage in connection with outbreaks of this disease do not offer a satisfactory solution of the problem. Udahl, Milks, and Williams have suggested that possibly no connection may exist between the feed and so-called forage poisoning.

The oat hay believed to be responsible for the outbreak of forage poisoning at the Griffith farm was not visibly contaminated with mould, but by plating the feed in dilutions, *Monascus purpureus* (Went) was isolated and artificially cultivated in large quantities for experimental feeding of large and small animals. The results, which have been reported,<sup>11</sup> are summarized as follows:

"*Monascus purpureus* (Went) grown under laboratory conditions had no etiologic significance in this outbreak of forage poisoning, since feeding large quantities over sufficient lengths of time did not produce the disease. We desire to present the evidence of the foregoing experiments as suggestive of the non-pathogenicity of *Monascus purpureus* (Went) when fed in large quantities, and when the products of its metabolism are injected intravenously. *Monascus*

<sup>10</sup> Tech. Bull. Mississippi Agr. Exper. Sta., No. 6, 1915.

<sup>11</sup> Himmelberger: Jour. Comp. Path. and Therap., 1915.

*purpureus* isolated from oats which had undoubtedly given rise to forage poisoning in horses and mules did not produce soluble or extracellular toxins *in vitro* on the cultural media employed, as shown by absence of clinical symptoms in the experimental animals."

Among the organisms isolated from the oat hay was a species like *B. coli*. Rogers, Clark, and Evans recently reported the prevalence of *B. coli* on American grains.<sup>12</sup> Feeding experiments with horses were conducted with the *B. coli*-like organisms.<sup>13</sup> No symptoms subsequent to the feeding of large quantities suggested that contamination with *B. coli* was fatal, or primarily involved in the animal losses, tho mild derangements were produced by feeding the organism disguised in wholesome feed.

Several micro-organisms isolated from the cerebrospinal system of affected animals have been described by many investigators as having an etiologic relation to this class of diseases. Hutyra and Marek<sup>14</sup> summarize these bacteriologic investigations as follows:

"Bacteriological investigations have, therefore, not as yet given perfectly satisfactory results, although it is probable that Siedamgrotzky and Schlegel, Johnne, Ostertag, Streit, Grimm, Christiani, Marcq, and possibly also Wilson and Brimhall, were dealing with the same organism which had in some way varied its characters somewhat. Further investigations are necessary to decide whether the cause of so-called Borna disease is always present in cases of cerebrospinal meningitis in the horse, and whether it plays any part in the production of the disease in other species, at least in a proportion of cases. The observations of Prietsch, Walther, Pröger, and Wilson and Brimhall, appear to indicate that this is the case. A solution is also required to the question as to what relationship exists between the organism described by Johnne and others and the diplococcus intracellularis of the human subject. According to Johnne the two may be distinguished by the fact that the organism which occurs in the horse may be present in the central nervous system without causing lesions, but simply an intoxication. According to Ostertag there is no connection between the two organisms. Christiani, on the other hand, was unable to find any differences between the streptococcus found by him and the diplococcus intracellularis of Weichselbaum."

Reichel informed us of a pathogenic coccus-like organism isolated from the cerebrospinal system of a horse that suffered from a disease resembling forage poisoning, as well as from a nonaffected horse that had been destroyed for student anatomic exercises. Our own efforts to isolate a pathogenic micro-organism from the encephalon of animals, naturally and experimentally affected, were not successful.

From the oat hay which formed the basis of our experiments a pathogenic micro-organism was isolated which grew readily under

<sup>12</sup> Jour. Infect. Dis., 1915, 17, p. 137.

<sup>13</sup> Graham and Himmelberger: Jour. Bacteriol., 1915, 1, p. 115.

<sup>14</sup> Pathology and Therapeutics of the Diseases of Domestic Animals, 1913, 2, p. 610.

laboratory conditions. A somewhat similar micro-organism has been isolated from the chicken excreta found in the oat hay, but its pathogenicity has not yet been established. This micro-organism was not isolated from 2 apparently wholesome forages examined bacteriologically, but from sorghum ensilage obtained from a farm where animal fatalities had occurred with symptoms resembling forage poisoning, a micro-organism with somewhat similar cultural characteristics was

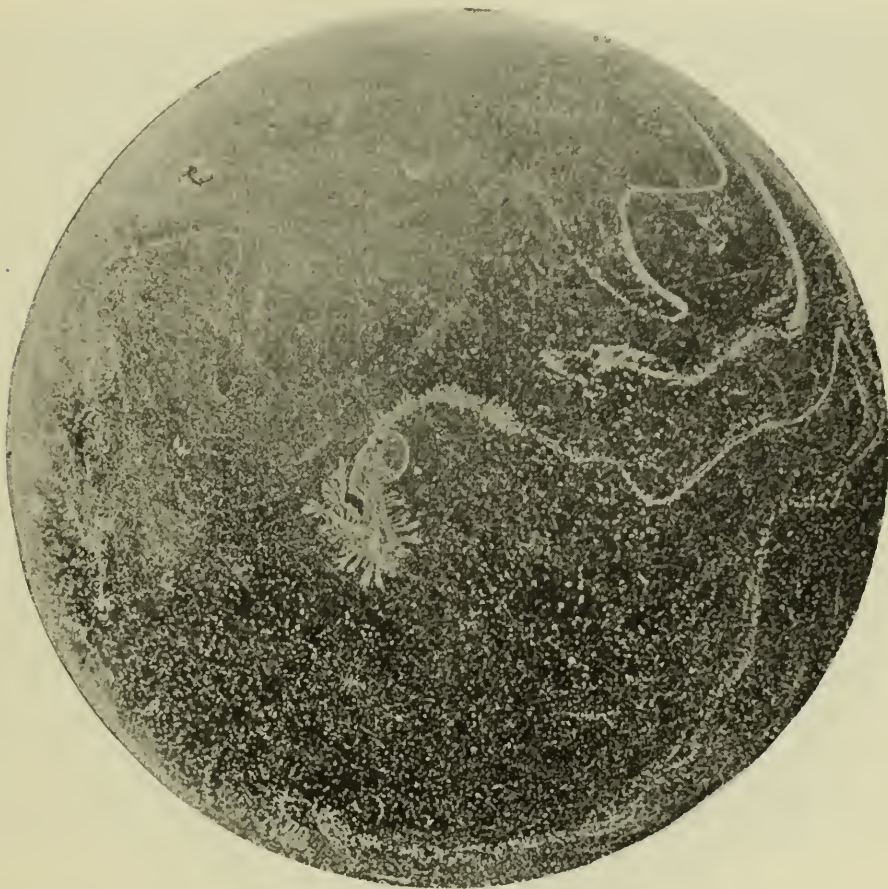


Fig. 1. Poured agar plate of the bacillus isolated from oat hay (Culture O<sup>1</sup> P<sup>2</sup>, 4 days old).

obtained. The micro-organism from the sorghum ensilage possesses pathogenic properties, as observed following inoculation of experimental horses.

The micro-organism isolated from the oat hay exhibited the following cultural\* and pathogenic characteristics:

\* The cultural characters here described were corroborated by Prof. R. E. Buchanan of the Iowa State College and Dr. I. J. Kligler of the American Museum of Natural History, working with subcultures submitted by the authors.

## MORPHOLOGY AND STAINING PROPERTIES

It is a bacillus, from 0.4 to 0.5 micron wide and from 1 to 2 microns long, occasionally longer, with rounded ends. In newly isolated cultures it resembles a coccoid bacillus. It occurs singly, chains of 2 bacilli being rarely found. It is motile, possessing flagella. Spores are produced, as demonstrated by heating a broth culture to 80 C. for 15 minutes and subculturing, as well as by staining reactions. It is stained by the ordinary aniline dyes, tho often unevenly with a tendency to grouping in the field. Gram-negative. The optimal temperature is from 35 to 37 C. It is aerobic, satisfactory growths not being obtained under strictly anaerobic conditions.



Fig. 2. Stroke cultures on an agar plate of the bacillus isolated from oat hay (Culture O<sup>1</sup> P<sup>2</sup>).

## CULTURAL CHARACTERISTICS

*Agar Plate.*—Small bluish-white colonies make their appearance in 24 hours as surface and frequently as sub-surface colonies in poured plates. Surface colonies increase in diameter after 2 or 3 days' incubation, and are oval or round, occasionally spreading over the surface. Rosette figures may be observed. Sub-surface colonies remain small. Under magnification of 15 diameters they are of a yellowish amber color. Not sensitive to variations in alkalinity or acidity of culture medium.

*Agar Slant.*—A thin growth along the line of inoculation is visible in 24 hours; isolated colonies at a distance from the line of inoculation are also common. As the growth becomes heavier it may show echinulate formations along the line of inoculation, more marked at the base. A white or amber tinge is observed in older cultures, and the growth may be continuous and compact, with a wrinkled surface. Growth in young cultures is of a butyrous-like consistency. An unpleasant odor is sometimes detected in old cultures.

*Gelatin Stab.*—Growth on the surface is abundant, developing faintly along the line of inoculation. Slight liquefaction observed after 1 week.

*Litmus Milk.*—Peptonization.

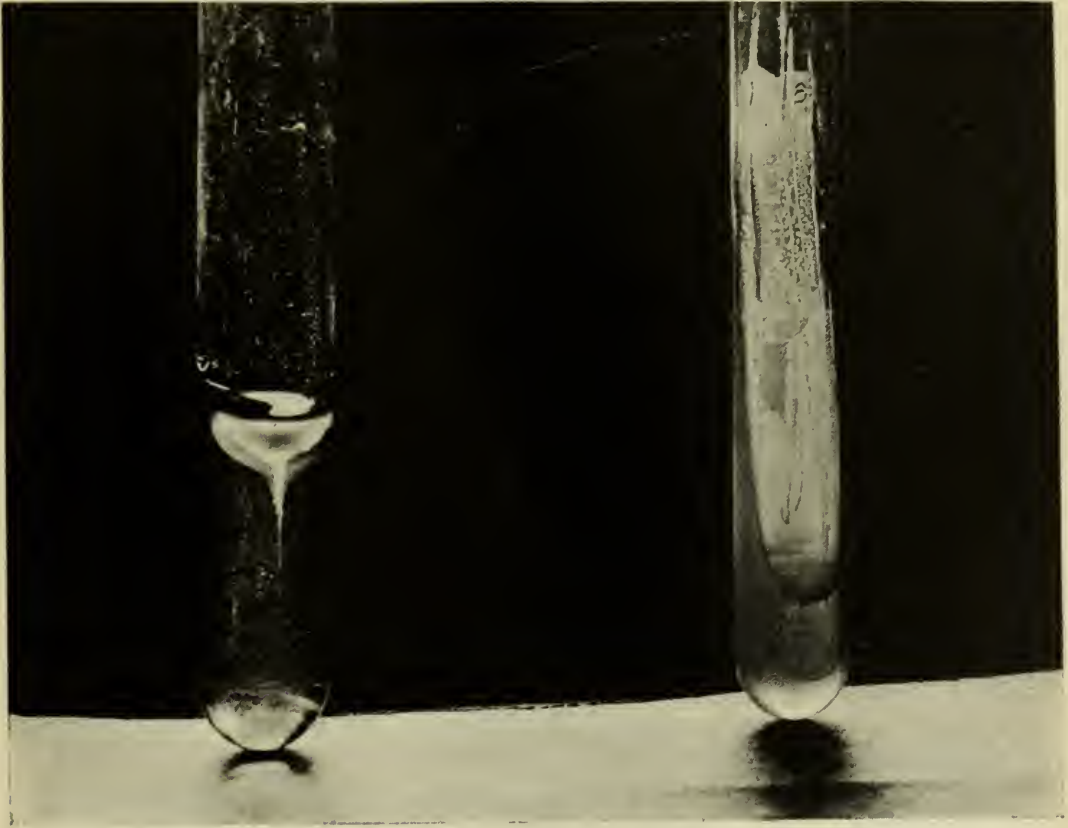


Fig. 3. Tube on left: Gelatin stab of the bacillus isolated from oat hay ( $O^1 P^2$ ) 10 days old. Tube on right: Agar-slant culture ( $O^1 P^2$ ) 1 week old.

*Gas-Production in Sugar Broth.*—No gas formed in maltose, raffinose, saccharose, rhamnose, dextrose, inulin, lactose, and mannite broth. No appreciable acid formed.

*Indol and Nitrite-Production.*—Indol-production could not be detected in Dunham's solution. Nitrates not reduced.

#### PATHOGENESIS

The bacillus is apparently nonpathogenic for rabbits, guinea-pigs, white rats, chickens, cats, dogs, and domestic swine, as observed from feeding and inoculation experiments to date. As very few of the large family of aerobic spore-forming bacteria have heretofore proved pathogenic, this organism was for a

time disregarded in our studies. It was later observed that horses, mules, cattle, sheep, and goats do not succumb to a single intravenous injection except in rare instances, tho symptoms of a transitory nature are generally observed, such as muscular tremors, dull, sleepy attitude, altered respiration. The growth on agar slants administered in sterile salt solution intravenously to horses results in marked symptoms in from 5 to 30 minutes. Repeated daily injections in horses are followed by nervous manifestations, marasmus, coma, and death. Cattle, sheep, and goats are more tolerant of similar injections, and some horses more so than others.

Broth cultures of the bacillus administered daily in doses of from 500 to 1,500 c.c. to horses by way of the mouth cause death in from 10 to 12 days. Beginning on or about the 5th day marked symptoms are observed from day to day, subsequent to each drenching, of muscular tremors, altered respiration, difficult deglutition, salivation, local paralysis, diffuse sweating, marasmus, prostration, and death. Broth cultures administered daily in 1,000 c.c. doses in the form of an enema to horses receiving wholesome feed cause prostration and death in from 7 to 12 days.

Cultures grown on Uschinsky's medium after passing through sterile cotton filters cause marked symptoms of distress when injected intravenously. Daily intrajugular injections of cultures obtained by filtration through Pasteur-Chamberland "F" and "B" candles, and sub-cultured to determine their sterility, result fatally in from 3 to 15 days. The symptoms manifested by animals so treated consist of a general depression, muscular tremor, salivation, difficult mastication, constipation, marasmus, incoordination of voluntary muscles, and inability to stand. While in a recumbent position the animals move their feet as if walking, or lapse into a condition of coma; during this time an indifferent nervous appetite may accompany a paretic-like condition of the pharynx. Death generally follows in from 1 to 5 days after the animal becomes unable to stand.

Passing through Pasteur-Chamberland "F" and "B" candles apparently modifies the virulence of the filtrate, tho heating to 80 C. in a hot bath does not destroy its effect entirely, as observed in animal inoculation. An active culture filtrate was tested chemically for protein by Dr. G. D. Buckner of the experiment station, with negative results, further confirmed by the absence of anaphylactic phenomena subsequent to guinea-pig and rabbit inoculations.

The symptoms observed in experimental horses following the injection of the sterile filtrate of this bacillus grown on Uschinsky's medium evidence the production of a toxin-like substance as an inherent character of the bacillus isolated from the oat hay, and the results from repeated administrations suggest a systemic cumulative action.

Repeated intravenous injections of the bacillus washed from agar slants, sterile filtrates of the culture on Uschinsky's medium, broth cultures by way of the mouth or in the form of enemas produced variable nervous symptoms and death in horses and mules. The fact that the oat hay, fatal when fed to horses, produced no effect when fed to small laboratory animals is in accord with the existing knowledge of forage poisoning; the nonpathogenicity for small animals of the bacillus here described therefore contributes to its significance as a pathogenic factor in this disease.

# SPONTANEOUS AMEBIC DYSENTERY IN MONKEYS\*

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This article records a spontaneous outbreak of a disease in monkeys in which the lesions corresponded closely to those found in amebic or tropical dysentery in man, and in which protozoal organisms occurred that had the structure and characteristics of those amebas generally considered the causative agents in human tropical dysentery.

This spontaneous outbreak of amebic dysentery is of special interest since there appears to be no record of a similar case, and because our knowledge of this disease in animals is very meager. It is probable that the affection was introduced with one or several of the imported monkeys, and conveyed to the healthy individuals kept in the same cage. The possibility of transmission of the ameba to human beings through such sources must be given cognizance, and, furthermore, our findings suggest that at times imported animals might be carriers of the parasites without disclosing any clinical evidence. This phase of the manner in which disease may be spread is now continuously gaining in importance.

Since the first ameboid protozoan was discovered by Roesel<sup>1</sup> (1755) numerous investigators have isolated this type of organism from various sources, differentiated the genus into a number of species, both parasitic and nonparasitic, and have attempted to classify the former as pathogenic and nonpathogenic. Lamb<sup>2</sup> (1859), Loesch<sup>3</sup> (1875), and Kartulis<sup>4</sup> (1885) observed amebas in fecal matter from dysentery patients, but Koch<sup>5</sup> (1887) demonstrated amebas in tissues undergoing ulceration, and was the first to establish a connection between amebas and the lesions of the disease known as tropical dysentery. Later investigators have confirmed the association of the parasites with dysentery, and have considered them the causative agent in this disease. Others have disputed the pathogenic rôle of amebas, asserting that the organisms can be demonstrated in the intestinal contents of a large percentage of men and animals in normal condition.

Tho the question of pathogenicity, and of permanent or facultative pathogenicity of certain species is undetermined, it is apparent from

\* Received for publication March 20, 1916,

<sup>1</sup> *Insecten-Belustigung*, 1755, 3, p. 101.

<sup>2</sup> *Beobachtungen und Studien aus dem Gebiete der pathologischen Anatomie und Histologie*, Teil 1, 1860.

<sup>3</sup> *Virchow's Arch. f. path. Anat.*, 1875, 65, p. 196.

<sup>4</sup> *Ibid.*, 1885, 99, p. 145.

<sup>5</sup> *Arch. a. d. k. Gsundtsamte*, 1887, 3, p. 13.

the specific character of the lesions in which these organisms are found that they are an important, if not the only, etiologic factor in the pathologic processes.

There are few data available on the spontaneous infection of animals. Artificial infections have been successful with various species of animals, especially with cats. However, in order to obtain such results material containing the spores or the encapsulated forms of amebas had to be fed, since the vegetative forms were invariably destroyed in the animals' stomachs. Spontaneous amebic dysentery complicated with liver abscess has been observed in a badger (*Meles taxus*) in the zoological garden at Cairo, and according to Strong, in an orang-outang in Manila. Castellani<sup>6</sup> also found a species of ameba in a liver abscess in a monkey. More recently Musgrave and Clegg<sup>7</sup> reported isolated cases in monkeys which were used for experimental purposes, and Macfie<sup>8</sup> a case in a monkey (*Ceropithecus petaurista*) which had served as a reservoir for human trypanosomes.

Aside from the amebic dysentery of man the only disease in which an ameba is supposed to be the etiologic factor is the so-called infectious enterohepatitis of turkeys, which, according to Theobald Smith,<sup>9</sup> is caused by *Ameba meleagridis*.

In the outbreak here described 8 animals out of a total of 15 exposed succumbed, and of 9 showing symptoms only 1 recovered.

#### THE CHARACTERISTICS OF THE AMEBAS

No special attempt was made to determine the species of the amebas concerned in this outbreak. The ameboid forms were especially numerous in liver abscesses, but could also be readily found in intestinal matter, the encysted forms being more numerous in the latter. In the vegetative stage they showed very active protoplasmic changes when taken fresh from liver-abscess pus, or from fecal material. This movement, which was apparent for a period of from 24 to 36 hours after the death of the animal, even without the aid of a warm stage, consisted of successive rearrangements of protoplasm without noticeable progressive movement across the microscopic field. Pseudopodia of various forms and lengths were extended from the surface and into these the protoplasm flowed, the nucleus changing its position simultaneously. On the completion of a movement the tendency was to return to a circular form about 30 microns in diameter. Absence of locomotion was probably due to the fact that the fluid medium in which the amebas were examined afforded no support for the pseudopodium.

<sup>6</sup> Parasitology, 1908, 1, p. 101.

<sup>7</sup> Rep. Philippine Is., Interior Dept., Bur. Govt. Lab., No. 18, 1904.

<sup>8</sup> Trop. Med. and Parasit., 1915, 9, p. 507.

<sup>9</sup> Bull. U. S. Dept. Agr., Bur. An. Ind., No. 8, 1895.

At times, on one side of the cell body, the protoplasm waved in one direction. The waves were started at one pole; a broad rounded pseudopodium was violently thrown out and whipped to the opposite pole, and the process repeated very rapidly for a period of several seconds or more. In some instances the protoplasm was clear and transparent, showing the circular nucleus plainly, but no granules or



Fig. 1. Amebas in vegetative and encysted forms. (a) Vegetative. (b) Encysted forms.  $\times 250$ .

foreign bodies. In other cases practically the entire interior was granular and appeared to contain several vacuoles, apparently noncontractile.

During the course of microscopical observations of fresh preparations from the several cases, the amebas gradually lost their motive power, assumed a roughly circular form, became more granular,

decreased in size, and appeared to be encysting. In the encysted stage 2 distinct areas were to be seen; the central portion showed a granular arrangement, was circular in outline, and surrounded by a clear transparent zone, which did not take up ordinary stains readily. In stained tissue sections containing the vegetative forms the nucleus appeared surrounded by a narrow stained band which separated it from the remainder of the cell body.

In feces examined after being voided by sick animals the vegetative forms present were sluggish, and the motility was of short duration, whereas material from liver abscess or intestinal exudate examined from 24 to 36 hours after the death of the animal was found to contain amebas in very active, tho short-lived, motility. This would indicate that the amebas passed with the feces were on the point of encysting in the lumen of the intestine, while those removed from the tissue after death were in an earlier stage of the life cycle, and had maintained themselves for a considerable period in the dead animal matter. The preponderance of encysted forms in the feces supports this view.

#### HISTORY OF THE OUTBREAK

In July 1915, 3 spider monkeys (*Ateles ater*) were received at the National zoological park from a dealer who had had them in his possession for some time. During September, 2 more were received, these from Colombia. There were 8 in the shipment, the other 6 being distributed among customers by the dealer. None has been reported sick except those at the National zoological park, and these together with the original 3 showed no signs of illness until December. On October 9, 2 spider monkeys were received from Salvador, and on October 26, 3 from Colombia. December 5, 5 gray spider monkeys, also from Colombia, were received. The animals were placed together in a large exhibition cage provided with facilities for exercising. One of the monkeys received October 26 was ill, showing symptoms, according to the keeper, similar to those later manifested by the others. It appears probable that this monkey carried the infection, altho the assumption can not be definitely proved.

The animals ranged in age from 2 to 5 years. In the normal state they were very active and while well filled out and muscular, they showed no tendency to fat-formation. These monkeys do not breed in captivity. Their natural habitat is in Central America, in the region extending from Nicaragua to Colombia, inclusive. They live in high trees and are found at any elevation between sea level and 7,000 feet altitude. In the wild state the food consists of fruits, insects, birds' eggs, young birds, leaves, bark, and seeds. At the National

zoological park the food consists of evaporated milk and raw egg daily, cooked rice, baked beef twice a week, wheat one day and sunflower seed the next, bananas, apples, beets, carrots, and sweet potatoes, both raw and cooked.

Since the date of the introduction of the disease among the monkeys is uncertain, the period of incubation cannot be defined. The first death occurred on November 21, 1 of the Salvador monkeys dying of nephritis, 44 days after arrival. The first death from amebic dysentery was on November 26, the animal that was sick on arrival being the first to succumb. Between November 26 and February 24, 8 animals died of dysentery; 1 had been very ill for a month and had apparently recovered, the only one to recover after showing symptoms of disease. Of the 5 monkeys placed in the cage on December 5 none contracted the disease, altho they were exposed for some time.

The floor and the interior of the cage, which were washed daily, were perfectly sanitary. However, there was ample opportunity for infection to spread from one to another through close association, and through contamination of food and water in the cage by the affected ones. Furthermore, it was observed by the attendants of the monkey house that the spider monkeys in that particular cage developed a depraved appetite for the feces which they evacuated, and as this is somewhat of an unusual occurrence among monkeys, it would readily explain the cause of the rapid spread of the disease.

#### TRANSMISSION EXPERIMENTS

To determine the relationship of *Ameba ateles* to the tropical dysentery of man, an attempt was made to transmit the disease to cats, since it is generally understood that these animals are susceptible to *Ameba histolytica*.

Two grown cats and 2 kittens were fed for 30 days with material from the intestines of monkeys dead of dysentery, and with feces from 2 sick monkeys. The material, which was known to contain the parasite, was fed while comparatively fresh, and also after it had been retained for several days at room temperature to promote encystment. Mixed with the food of the animals it was taken readily. The cats remained healthy, showing no indication of diarrhea, except for an occasional temporary looseness in 2 of them, apparently from other causes. Amebas were not found in the feces and at the expiration of 30 days of feeding, autopsies failed to reveal any lesions in the intestine or in other internal organs.

These negative transmission experiments suggest that the parasite found is of a different species from that in man, and that it is specific for the spider monkey, particularly since the cats cohabitated in the

same cage with sick monkeys, and were afforded ample opportunity for the ingestion of encysted forms of the parasite. The results, however, do not warrant any conclusions as to the pathogenicity of this parasite in cats, since failure of transmission to a limited number of animals is not sufficient evidence, various investigators having found that the transmission of amebic dysentery to cats is not invariably successful. No other species of monkeys kept in other cages of the monkey house became affected, altho they were in close proximity. However, the exposure was likely very slight. There is always a possibility, however, of parasitic organisms' being carried by attendants on cleaning utensils, unless unusual precautions are taken.

#### SYMPTOMS

The first symptom noticed was a tendency to assume the resting attitude. The affected animal sat upright with the head pressed down between the hind legs and the long tail curled around the body. Marked dejection, stupidity, and lack of interest were evidenced. The temperature remained practically normal and the appetite, while diminished, was fairly good up to the time of death. The animals decreased in weight during the period of illness, but were not markedly emaciated.

The principal symptom was a severe diarrhea. The feces were usually of a fluid consistency, yellowish-gray in color, and fetid, containing at times considerable mucus with yellowish flakes. In the more acute cases the excrement was sometimes blood-stained. At first the diarrhea was intermittent, fairly well-formed stools being passed during intervals, but later the diarrhea persisted till the end. In several cases symptoms were apparent for only 2 or 3 days before death, while in other cases the animals showed signs of illness over a period of from 2 to 4 weeks, at times appearing to have recovered somewhat, only to relapse and die suddenly.

#### GROSS LESIONS

In the 8 cases in which autopsies were made the cecum and colon were invariably the seat of pathologic changes, the rectum being involved to a greater or less degree. No lesion could be detected macroscopically in other portions of the gastro-intestinal tract. Even in the most gross cecal infection the amebic invasion did not pass beyond the ileocecal opening. Liver abscess was present in 2 cases. The mesenteric lymph glands in the region of the colon were in some instances found to be enlarged and edematous.

In the large intestine, which was approximately 18 inches long in the monkeys under discussion, the lesions were pronounced and distinctive. The appearance of the grossly affected portions of the mucosa was that of a dense corrugated mass of grayish-white mealy-looking necrotic matter. In the advanced cases where the disease had evidently been of long duration, the process had spread to practically the entire membrane. In less affected intestines numerous small ulcers, varying in size from 1 mm. to 1 cm. in diameter, were found, well separated from each other. These presented raised irregular borders on which a line of congestion could readily be traced. The depressed centers were occupied by a fairly closely adhering mass of necrotic exudate, which also



Fig. 2

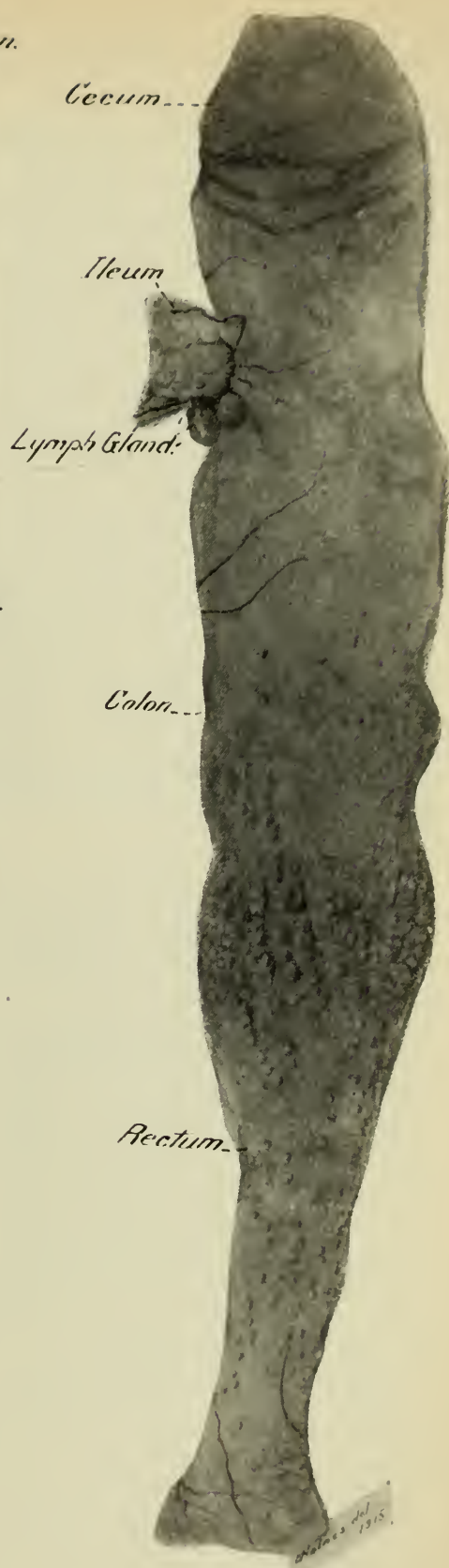


Fig. 3

Fig. 2. Large intestine of a monkey illustrating extensive exudate on the surface of the mucosa of cecum and colon, and ulceration of the rectum. Extensive involvement with diversified and confluent lesions. About one-half natural size.

Fig. 3. Large intestine of a monkey illustrating ulceration of colon and rectum. Cecum covered by a catarrhal exudate. Lesser involvement with ulceration predominating. About one-half natural size.

covered the surface of the ulcer to a height of from 2 to 3 mm. Affected portions of the intestine not marked by ulceration showed a thick covering of an amorphous detritus on the surface of the mucosa. The thickness of the exudate in severe cases gave the cecum and colon a dilated appearance and a semirigidity. The material composing the exudate could readily be scraped off the surface, appearing as a flaky mass easily broken up into fine particles; however, on its deep border it had more of a diphtheritic consistency and had a tendency to remain adherent to the neighboring tissue. Lines or points of deep congestion, or hemorrhage, were of frequent occurrence in the affected parts.

The deep ulcers penetrated beyond the submucosa, but no indication of the condition in the lumen of the intestine was apparent from the peritoneal surface, save where dilation or rigidity of the wall was present.

Abscess of the liver was associated with the 2 cases showing the most extensive intestinal lesions. In one case only 1 abscess was present, while in the other 5 fairly large abscesses and 2 smaller ones were observed. They were characterized by the absence of a well-defined capsule except at the point where the capsule of the liver was in apposition, at which a marked thickening of the latter had occurred. The borders were surrounded by a zone of small necrotic areas of irregular outline. The abscesses were deeply extended and spread out within the interior of the lobes. Their interiors held the remains of liver structure which had not yet undergone complete disintegration, in a network in which pockets and channels were conspicuous. These openings were filled with a grayish-white fluid pus which exerted an appreciable pressure on the abscess border. In fresh preparation of the pus the microscope disclosed numerous amebas showing protoplasmic movement.

#### HISTOLOGY

*Intestine.*—The intestinal wall showed variable changes according to the degree of involvement. Ulceration of the mucosa was prominent in places, the position of the glands of Lieberkuhn being occupied by detritus, in which were observed disintegrating mucous cells, lymphoid cells, and amebas. The thickness of the necrotic exudate covering the affected areas was about twice the thickness of the normal mucosa. The necrotic foci were at times partially encapsulated by fibrous tissue and lymphoid cells, especially when the submucosa was involved. The tissue surrounding the foci did not appear to be greatly damaged, altho the immediately adjacent cells stained more diffusely and were more or less separated, indicating an edematous condition of the part. A striking feature of some pieces of cecal tissue was the fact that altho there was a dense exudate covering the entire mucous membrane, the latter revealed marked changes only in its surface cells. These were disintegrated and separated from the basement membrane here and there, while at other points damage was evidenced only by the difference in staining properties. The submucous, muscular, and serous layers of the intestine were not particularly affected except in areas of deep ulceration.

Superficial ulcers manifested a tendency gradually to destroy the adjacent mucosa without causing any accumulation of inflammatory cells, and without a trace of the formation of fibrous tissue. This probably indicated rapid advance without a marked stimulative effect on the protective forces of the system.

In the case of deep ulcerations the necrotic process had involved not only the mucosa, but had penetrated also through the submucosa, muscularis mucosa, and circular muscular layer, and produced distinct degenerative changes in the longitudinal muscular layer. Around the base of the deep ulcers small mono-

nuclear leukocytes were massed in large numbers. A distinct characteristic of deep ulcers was the formation of a dense fibrous wall undermining the mucosa to a considerable distance, and causing a thickened or raised appearance in the ulcerated area. The mucosa in the undermined region had in areas almost lost its identity, and tho in adjoining areas the structure was preserved, disintegration changes were quite apparent. The center of the ulcers consisted of an amorphous mass, breaking through the surrounding fibrous wall and resting in the circular muscularis, the surrounding fibers of the latter showing marked degenerative changes to a considerable depth.

In some instances the mucous membrane surrounding the mouth of the ulcer presented a punched-out appearance; in others the membrane was shrunk or erased on one side, while seeming fairly normal on the opposite side in the histologic section.

The histologic picture was characterized by the absence in large part of micro-organisms other than amebas; the absence of the types of leukocytes usually found present in microbic infections; the failure of fibrous capsule to form, except in the submucous layer, where the thickening was marked when ulcers extended into this coat; and by the dense covering of detritus at points where little harm had been done to the underlying mucosa. The absence of pronounced congestion except in superficial areas where microbic activity was more or less evident, was also characteristic, and suggested the specific nature of the amebic lesions.

Amebas were disposed through the proliferated material and were to be found in the disintegrating tissue surrounding the ulcers and in the glands of Lieberkuhn in affected areas.

*Liver.*—The tissue bordering a hepatic abscess presented numerous necrotic foci varying in size from a 4- or 5-cell area to a macroscopic abscess. The tendency in the larger foci was toward encapsulation. A considerable number of lymphoid cells were observed in the fibrous structure of the wall. The encapsulated mass represented a homogeneous material in which a few lymphoid cells and also amebic forms might be found.

The picture was characterized by the varying degree of the degenerative processes in the infected areas. At points where several amebas were lodged no changes were observed in the staining properties of the surrounding liver cells, altho a distinct separation of the cell columns was evidenced. At points where degenerative changes had progressed to a detritus-formation the amebas were arranged around the periphery of the foci and extended in places beyond the zone into the normal structure. In the case of the more advanced foci a well-defined wall of fibrous tissue had formed, lymphoid cells being present to some extent in the outer border, and at times in the central mass. In some instances the encapsulation was marked and apparently had arrested the amebic progress, as no amebas were visible in the foci.

Between the foci of degeneration the liver cells did not appear to have become at all involved except in the case of those forming the surrounding layer, and even here there was no marked change unless the area was partly inclosed by 2 or more foci.

Isolated amebas were found in the midst of normal hepatic cells at a distance from visible lesions, but it is probable that these had migrated from closer lesions not revealed in the particular tissue section.

Congestion was in evidence, the small vessels in the invaded areas showing a noticeable fullness. However, no hemorrhages were observed in the tissue examined.

## CASE 1

Carcass somewhat emaciated. Left lung congested in anterior lobe. Right lung slightly congested. Considerable clotted serous exudate in pleural cavity. Spleen enlarged to twice its normal size.

The anterior surface of the liver contained an abscess covering about one-third the area of the principal lobe. The apex of the abscess pointed to the diaphragm, to the fibrous part of which it was firmly attached over an area three-quarters of an inch in diameter. Around this adhesion the abscess-formation was well marked beneath the capsule, which had a thickened appear-

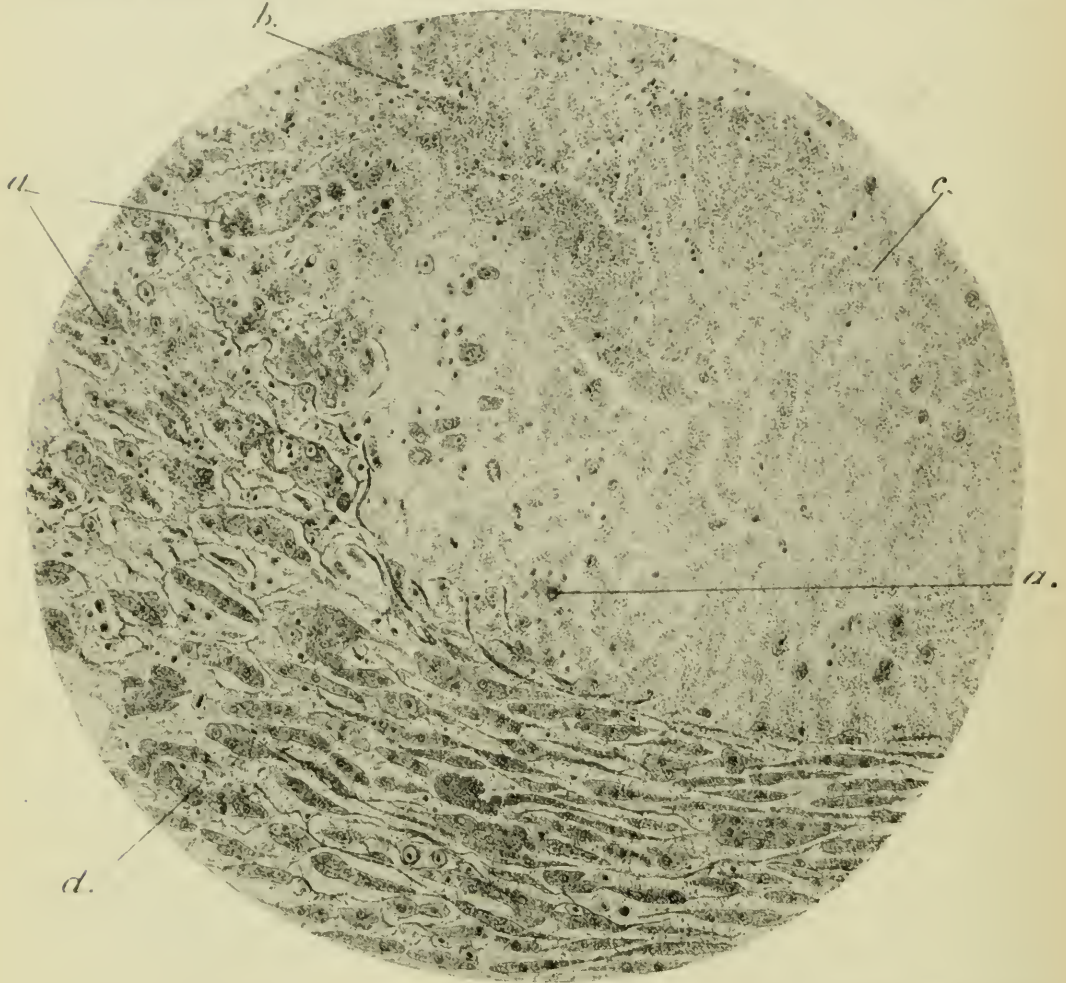


Fig. 4. Section of the liver of a monkey illustrating the edge of small necrotic foci. (a) Amebas. (b) Lymphoid cells. (c) Necrotic detritus. (d) Liver cells.  $\times 125$ .

ance. Surrounding the border of the large abscess were numerous small necrotic areas varying in size from one to several millimeters in diameter. When the adherent diaphragm was cut away, a grayish-white pus was released, proceeding from several well-defined channels within the degenerated liver structure. Microscopical examination of this pus in the fresh state revealed numerous amebas undergoing active changes in the form of their protoplasm. On cut section the abscess proved to be deeply extended in the body of the lobe, undermining the superficial structure.

Entire cecum and nearly the entire length of the colon covered on the surface of the mucosa with necrotic material of a grayish-white color. Exudate from 2 to 3 mm. in thickness, of mealy appearance, and thrown into longitudinal and horizontal folds; of a granular consistency in its deepest layer, approaching the diphtheritic type of exudate. The extensive necrotic formation gave the intact cecum and colon a distended appearance and semirigid position, which directed attention to their abnormal condition before an incision through their walls was made. The process did not extend to the ileum, altho the ileocecal valve was involved. In the posterior portion of the colon and the anterior part of the rectum ulcers from one to several millimeters in diameter were found, isolated and numerous. These ulcers had a raised hemorrhagic border, were irregular in shape and covered by the same type of exudate as that previously described. Hemorrhagic points observed here and there throughout the affected area.

Microscopically the fresh cecal material showed the presence of motile and encysted amebas.

#### CASE 2

Carcass in fair condition. No lesions apparent in internal organs other than cecum, colon, and rectum.

Cecum and colon dilated and fairly rigid. On being incised the mucous membrane found in a condition similar to that of Case 1, the thickened mass of exudate being strikingly prominent in spots, but not as widely distributed over the surface. Congestion relatively marked over the entire area of the mucous membrane of the colon and rectum. Mesenteric glands in the affected region edematous.

Amebas present in the cecal material.

#### CASE 3

Carcass emaciated. Internal organs other than large intestine and liver normal.

Distributed through the liver were 5 abscesses varying in size from one-half to three-quarters inch in diameter, and 2 smaller abscesses. These which did not appear to be well encapsulated, were surrounded by zones of minute areas of degeneration. Capsule of the liver noticeably thickened over the abscesses, preventing rupture into the abdominal cavity.

Mucous membrane of the cecum and colon ulcerated and covered in large areas by the heavy uneven-surfaced necrotic material. Numerous small ulcers with raised congested edges scattered in the less affected regions. Mesenteric lymph glands enlarged and congested.

#### CASE 4

Carcass somewhat emaciated. Internal organs other than large intestine apparently normal.

Mucous membrane near the blind end of the cecum thickened, giving indication of early ulcer-formation. Surface covered by a thin deposit of necrotic matter. Mucosa of the posterior portion of the colon covered with small ulcers 3 mm. in diameter, isolated from each other and showing prominent raised hemorrhagic borders. A dense grayish-white exudate capped each ulcer. Between the ulcers mucosa normal. Anterior extremity of the rectum also ulcerated, but ulcers less numerous and more widely separated. Several healing ulcers obscured, raised above the surrounding membrane and containing a central

depression. Some exudate material still clung to the surface. Mesenteric glands along the affected area edematous.

Amebas present in smears of cecal material.

#### CASE 5

Animal received from the zoological park in a sick condition; droopy; sat huddled up and inattentive. Diarrhea a prominent symptom, the feces liquid with small amount of solid material, and yellowish-gray in color; amebas present. Appetite good until the day before death. Depression became more pronounced, death following a period of 12 days' noticeable illness.



Fig. 5. Vertical longitudinal section of colon illustrating deep ulcer. (a) Ulcer. (b) Mucosa. (c) Dense fibrous tissue replacing submucosa. (d) Circular muscular layer. (e) Longitudinal muscular layer. (f) Serous layer. (g) Detritus.  $\times 7$ .

Fig. 6. Vertical longitudinal section of colon illustrating edge of deep ulcer. (a) Amebas. (b) Necrotic detritus. (c) Mucosa. (d) Fibrous wall. (e) Circular muscular layer. (f) Longitudinal muscular layer. (g) Serous layer.  $\times 30$ .

Carcass in fair condition. The cecum and colon showed corrugation of the mucous membrane in patches associated with inflammatory areas. Ulceration and exudate-formation in places, but not as extensive as in the previous cases. Exudate material revealed amebic forms.

#### CASE 6

Carcass emaciated to some extent. Cecum, colon, and anterior rectum presented the characteristic ulceration of the mucosa observed in previous cases. The lesions were developed in patches and in areas of diffuse single ulcers. Amebas in the cecal exudate.

#### CASE 7

Carcass showed no fat. All organs except large intestine apparently normal. Cecum, colon, and first portion of rectum showed diffuse areas of congestion associated with small patches of yellowish exudate, giving the appearance of dull sulfur-like deposits scattered over the surface of the mucosa. The intestine was practically free from feces, but a small amount of viscid material coated the cecum, the wall of which appeared thickened, tho not thrown into ridges. Membrane of the colon and rectum smooth with no marked ulceration, but with numerous congested areas and necrotic patches.

The contents of the intestine, especially the sulfur-like grains of exudate, showed numerous amebas in active protoplasmic movement, also encysted forms. No progressive movement across the field of the microscope, but pseudopodia of different forms thrown out, the nucleus changing its position rapidly as the protoplasm flowed into the protruded capsule.

#### CASE 8

Animal had been sick for 2 weeks before being brought to the laboratory. Condition grew worse daily; the feces were liquid and became blood-stained at times several days before death, which occurred in 12 days. At autopsy considerable emaciation evidenced.

All internal organs apparently normal except the large intestine; diffuse ulceration throughout its length. Colon conspicuous by its hemorrhagic appearance, almost the entire surface of the mucosa being deeply reddened. This inflammatory condition entirely superficial, involving the mucous membrane alone. The picture represented extreme congestion, the earlier cases being of the exudative type and showing congestion only in small lines around ulcers, or in small areas. In this case the ulceration was not marked and little exudate was apparent.

Altho motile forms of the ameba were numerous in fecal material for several days before death, only encysted forms were observed immediately after death.

## THE STRENGTH AND COMPOSITION OF BLACKLEG VACCINES \*

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A report of investigations carried out by this department on the strength and composition of some of the most widely known blackleg vaccines is here presented. One commercial vaccine consisting almost entirely of pathogenic spore-forming organisms, not *Bacillus chauvæi*, was subjected to a special study, since the strength of this vaccine differed markedly from that of all other vaccines.

The experiments were undertaken to determine, if possible, the fundamental reasons for so many unsatisfactory results from the use of blackleg vaccine. Complaints had been received concerning all vaccines in common use. Part of these involved losses of calves immediately after vaccination, others death from blackleg in spite of numerous vaccinations. The reports of nonprotection were especially numerous when the station followed the Kitt method of attenuating in streaming steam. When the vaccine was attenuated by dry heat, and hence was stronger than the steam-attenuated material, several notices were received of the vaccine's having apparently produced blackleg. From reports covering a period of 6 years the conclusion was drawn that there is no vaccine in use which does not justify serious complaints from one or both of these causes. After consideration of the opinions of various authorities we decided to measure the strength of the various blackleg vaccines on guinea-pigs.

Foth,<sup>1</sup> in speaking of his pure-culture vaccine (which would undoubtedly be far safer than any other form of vaccine because of the absence of contamination, which is known to increase the virulence of blackleg material, and also because of its fine trituration, making it even and regular in strength), states: "The spore-containing vaccine for direct subcutaneous injection should by no means be more than  $\frac{1}{2}$  of the minimum lethal dose for guinea-pigs, when given subcutaneously in the ear. If the inoculation is performed in the neck instead of the ear, it seems advisable to reduce the dose to  $\frac{1}{3}$  the minimum lethal dose for guinea-pigs. Due to the great expense inherent we have not been able to obtain data sufficient to make positive statements."

\* Received for publication March 20, 1916.

<sup>1</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1911, 10, p. 1.

Kitt<sup>2</sup> states that when the steam-attenuated vaccine was injected in doses of 30, 50, and 100 mg. it was not fatal for guinea-pigs, and the only reaction in cattle vaccinated subcutaneously with from 20 to 100 mg., was local edema, hardly noticeable.

In a personal communication Dr. J. R. Mohler of the U. S. Bureau of Animal Industry wrote in 1914 as follows: "The test used by the government bureau in the testing of their vaccine is that 3 guinea-pigs receive  $\frac{3}{4}$  of the usual dose for calves, 3 guinea-pigs receive  $\frac{1}{2}$  of the usual dose for calves, and 2 guinea-pigs receive  $\frac{1}{3}$  of the usual dose for calves. If none die, the vaccine is good. A reaction is desired." In Dr. Mohler's opinion the variation in individual susceptibility of guinea-pigs plays a great part in preventing progress in establishing a standard.

In striking contrast with these views is the statement by King<sup>3</sup> that "when results are correct those [guinea-pigs] which receive  $\frac{3}{4}$  the usual dose, should die, those receiving  $\frac{1}{2}$  should show symptoms and recover, while those injected with  $\frac{1}{3}$  should remain normal."

#### TEST OF BLACKLEG VACCINES ON GUINEA-PIGS

Blackleg vaccines from a number of laboratories in this country and Europe were tested. The values given represent approximately the average fatal dose for a guinea-pig weighing from 9 to 13 ounces. The commercial vaccines are designated by letters, A, B, C, D, and E.

#### POWDER VACCINES

Bernese, powder, single.....	100 mg.	} fatal to guinea-pigs
Bernese, (1 guinea-pig) .....	40 mg.	
Lyonese, single .....	150 mg.	
B. A. I., Lot A (single).....	100 mg.	
B. A. I., Lot B (single).....	20 mg.	

Ten mg., after filtration, is the dose for yearlings. The Bernese vaccine was obtained by Dr. D. A. Guillebeau of Berne, Switzerland; the Lyonese was obtained through a Paris distributor; and the B. A. I. vaccine is the government vaccine, distributed by the U. S. Bureau of Animal Industry, Washington, D. C.

#### PELLET VACCINES

E vaccine.....	3 doses not fatal, 5 occasionally so.
D vaccine.....	3 doses not fatal; lethal dose not determined.
C vaccine.....	3 doses not fatal; lethal dose not determined.
A vaccine.....	3 doses not fatal, except of 1 serial, of which 1 or 2 doses were fatal. Severe losses occurred in several herds following the use of this serial of A vaccine.
B vaccine.....	1 dose regularly fatal.

The results of the tests carried out on the various pellet vaccines are here given in detail. It was difficult to ascertain just where the M.L.D. lay in many cases on account of the death of the guinea-pigs from

<sup>2</sup> Centralbl. f. Bakteriöl., 1888, 3, p. 572.

<sup>3</sup> Quoted by Marshall, Microbiology, 1912, p. 472.

contaminating organisms when several pellets were given; this was not the case, however, with A single vaccine of which from 1 to 2 pellets were fatal, as this was practically free from contaminating organisms.

*B Single Pellets.*—Of a lot of 16 guinea-pigs receiving 1 pellet each of B. single vaccine, 2 lived and 14 died. B vaccine, single, was tested more thoroughly than any of the others. The tests included several different lots of vaccine.

*B Double Vaccine Pellets.*—Two pellets of the first of the double killed a guinea-pig. The second of the double was not fatal for a guinea-pig in small fractional doses of  $\frac{1}{4}$  and  $\frac{1}{8}$  pellet by weight, but was fatal in 1 of 2 guinea-pigs in a dose of  $\frac{1}{2}$  pellet.

*C Pellets.*—A European single-pellet vaccine was found to be not fatal in guinea-pigs in doses of from 1 to 3 pellets, and occasional tests showed 5 to be safe. These pellets were of larger size and almost twice the weight of the B-pellet vaccine.

These vaccines, with the exception of B and C vaccine, were apparently made from blackleg-affected muscle.

*E Single Pellets.*—The guinea-pigs that received 1 pellet each, remained alive. Others that had received the same dose died from causes other than blackleg, no blackleg organisms being recovered.

*A Single Pellets.*—One pellet was not fatal for guinea-pigs.

These data show that the guinea-pig M.L.D. of the powder-form vaccines tested, varies from 20 to 150 mg., and that about 100 mg. are required of most of the vaccines to kill a guinea-pig.

These vaccine powders are mixed with water and filtered before use, 10 mg. of powder being extracted for each calf to be vaccinated. To what extent the filtration lessens the virulency has not been exactly determined. In some of these experiments the filtered vaccine was much weaker than the emulsion before filtration; in a few cases the filtrate was almost as virulent as the mixture before filtration. Even when making no allowance for weakening due to filtration, at least 2 doses of the strongest powder-form vaccine tested were required to kill a guinea-pig.

None of the pellets tested, with the exception of B, was fatal for guinea-pigs in doses of 3 pellets, the one serial of A vaccine, as already noted, being excepted. These observations on the use of A vaccine, as well as a series of experiments carried out at the station, have shown that serious vaccination losses may frequently follow the use of a black-leg vaccine on calves when the strength of the vaccine is such that one or two times the quantity administered to each calf is sufficient to kill a guinea-pig with blackleg. It will be noted that 1 pellet of B single vaccine was regularly fatal for guinea-pigs. In spite of this fact field observations indicate that very few vaccination accidents are attributa-

ble to B vaccine. This was at first considered remarkable, but on closer investigation it was shown that the virulence exhibited in guinea-pigs by the B vaccine was not due to *Bacillus chauvæi* but to another pathogenic spore-forming anaerobe, provisionally referred to as the bacillus of pseudoblackleg.

The presence of this pseudoblackleg bacillus in the B vaccine produces certain marked differences between this and the vaccine containing the blackleg bacillus, as shown here:

B VACCINE	VACCINES MADE FROM BLACKLEG MATERIAL
One dose of single regularly fatal to guinea-pigs.	One to 3 doses not fatal to guinea-pigs, Aerobic contamination; C pellets generally sterile.
Sterile, i. e., no aerobic organisms.	Irregular in strength.
Regular in strength.	Rapidly loses strength with age.
Does not rapidly lose strength with age.	Dangerous in doses of 1 pellet when of the same strength as B pellets.
Safe in doses of several (5 to 7) pellets per calf.	Many losses following field vaccination.
Very few losses reported immediately following field vaccinations.	Reports received at this station covering B. A. I. vaccine show fairly good field protection.
Reports covering its use in the field show poor protection.	Blackleg organisms recovered from nearly all pellets tested.
No blackleg organisms recovered from pellets or animals killed with same, tho numerous attempts were made.	After immunization to one strain of blackleg the guinea-pigs have been immune to all other strains as far as tested.
Pellets produce death in guinea-pigs that are thoroughly immune to blackleg.	Foth's blackleg serum protects against all strains of blackleg tested.
Foth's blackleg serum does not protect against this pill or against the pseudoblackleg cultures.	

These important findings in regard to B vaccine prompted a brief examination of the other vaccines (Table 1).

TABLE 1  
BACTERIOLOGIC TESTS OF BLACKLEG VACCINES

Vaccine	Aerobic Organisms	Blackleg Organisms	Anaerobic Organisms Besides Blackleg
B single pellet { 1st pellet { double vaccine { 2nd pellet	Absent .....	Absent .....	Pseudoblackleg
E single pellet.....	Present .....	Present .....	None
C single pellet.....	Present in some cases	Present (?) .....	
D single pellet.....	Present in all cases...	Present .....	
A single pellet.....	Present .....		
Foth's pure-culture vaccine...	Absent .....	Present in all cases...	
Bureau An. Ind., powder....	Present .....	Present in all cases...	
Lyonese powder, single.....	Highly contaminated..	Present .....	
Bernese powder, single.....	Highly contaminated..		

B VACCINE AND THE ISOLATION AND DIFFERENTIATION OF THE PSEUDO-  
BLACKLEG BACILLUS

To isolate the pseudoblackleg bacillus guinea-pigs were inoculated with 1 pellet of the B single vaccine. They succumbed within from 48 to 72 hours with a disease somewhat resembling blackleg. The essential differences between pseudoblackleg lesions and those of true blackleg were a heavy gelatinous exudate, and a slightly lighter discoloration of the affected parts in pseudoblackleg; in true blackleg the muscles were blacker, more spongy, and drier. The odor of blackleg was absent. The greenish cast over the affected areas, which resembled an advanced putrefaction, was typical of pseudoblackleg at the time of death. It was never noticed in pure blackleg, except from 12 to 18 hours after death, and then only in very warm weather.

The pseudoblackleg bacillus was the only one that could be cultured both from the pellets and from the animals inoculated with the pellets. Its morphology resembled that of the blackleg bacillus somewhat. It was a slightly thicker rod and had less tendency toward the early formation of spores and clostridia. The shape of the spores was slightly more oval, and larger staining areas surrounded the spores in their earlier formation than in blackleg. However, these differences were so nearly approached by nontypical organisms of each species that their diagnostic value is not absolute. The colonies in liver agar were irregular in outline, the blackleg colonies regular. The organism grew more luxuriantly than the blackleg organism, and under less strict anaerobic conditions. The gas formula in 4% glucose broth was  $2\text{CO}_2/\text{H}$  to  $3\text{CO}_2/\text{H}$ ; the gas formula for the pure blackleg cultures always showed more H than  $\text{CO}_2$ . Pseudoblackleg blackened ferrous-sulfate agar, gave off a putrefactive odor, and was pathogenic in rabbits.

## THE PATHOGENICITY OF PSEUDOBLACKLEG IN RABBITS

Ten rabbits of approximately the same weight and condition were selected and 5 of them inoculated intramuscularly with a culture of pure blackleg, the other 5 receiving, in the same manner, an equal dose of a pure culture of the pseudoblackleg organism. The cultures had been grown in the same media and under similar conditions. The pseudoblackleg culture had been isolated from a guinea-pig which had died from a B pellet. The culture of pure blackleg was of such virulence that 0.1 c.c. was uniformly fatal for guinea-pigs.

Three rabbits received 0.5 c.c. each of pure blackleg culture, No. 278, one rabbit received 1 c.c. and one received 1.5 c.c. None of the rabbits died or became even noticeably ill, while of the 5 rabbits receiving the same dose of pseudoblackleg only one remained alive. Pseudoblackleg organisms were recovered from the affected muscles of the rabbits that died. The B vaccine pellets were fatal for rabbits in 2 of 3 instances, when 2 pellets were given intramuscu-

larly in the thigh. Two rabbits similarly injected with 1 pellet each, remained well.

That the rabbits were as susceptible to pseudoblackleg as the guinea-pigs is evident if we consider the difference in weight between the two species. The lesions resembled those in the guinea-pigs, and might easily have been mistaken for the typical blackleg found in calves. The blackleg cultures and the pseudoblackleg cultures used in this experiment both killed guinea-pigs in doses of from 0.03 to 0.1 c.c., so that the different results with rabbits were not due to a difference in virulence between the cultures, but to fundamental differences in their pathogenic properties.

Inasmuch as the pseudoblackleg organism differed from the pure blackleg organism in cultural and pathogenic properties, experiments were undertaken to ascertain whether it possessed any power to immunize against *Bacillus chauvæi*. Numerous attempts were made by means of pure cultures, by extracts, and by fractional parts of pellets to render guinea-pigs immune to pseudoblackleg. (The records of experiments concerning the attempted immunization of calves against this vaccine will be published shortly.)

In most instances the guinea-pigs died after the 2nd or 3rd injection, and only a few were immunized against several fatal doses of pseudoblackleg. These guinea-pigs, immune to pseudoblackleg infection, readily succumbed to blackleg infection when tested with pure cultures of *Bacillus chauvæi*.

Since such difficulty was experienced in immunizing guinea-pigs against pseudoblackleg, another series of experiments was carried out in which guinea-pigs were immunized against various strains of pure blackleg and, subsequently, inoculated with pseudoblackleg cultures or B pellets.

The minimal lethal doses for guinea-pigs of the various cultures and vaccines used in this experiment were as follows:

Bernese vaccine .....	100 mg.
Lyonese vaccine .....	150 mg.
B. A. I. vaccine.....	100 mg.
Culture 219.....	from 0.02 to 0.1 c.c.
Culture 306 .....	0.1 c.c.
Culture 298.....	from 0.05 to 0.1 c.c.
Culture 278.....	from 0.03 to 0.1 c.c.
Pseudoblackleg .....	0.1 c.c.

The lower figures were not always fatal, the M.L.D. varying from 0.02 to 0.1 c.c., which was fatal in over 95% of the cases. These figures are based on the results obtained in several hundred guinea-pigs. All strains of pure blackleg investigated had a guinea-pig M.L.D. constant within these limits when cultures were grown in a slightly modified form of von Hibler's brain medium.

Guinea-pigs were made highly immune to blackleg by means of blackleg vaccine and large doses of pure cultures; some received 4 doses at regular intervals during a period of 2 months, the last dose of pure blackleg culture representing 2.5 times the certainly fatal dose. Twenty-six days later they were inoculated with one uniformly fatal dose of pseudoblackleg, and all promptly succumbed to infection by pure cultures of pseudoblackleg isolated from B-vaccine pellets. Various blackleg vaccines, both domestic and foreign, including Foth's pure-culture vaccine and filtered blackleg muscle juice, were used to immunize the guinea-pigs so as to avoid the possibility of their not being immune to various strains of blackleg.

Guinea-pigs which had been similarly immunized against blackleg vaccine and cultures so that they would withstand several fatal doses of pure cultures of blackleg, were inoculated with 1 pellet each of B single vaccine, with fatal results showing that guinea-pigs highly immune to blackleg were not immune to the pseudoblackleg cultures or pellets.

The failure of the blackleg-immune guinea-pigs to withstand 0.1 c.c. of the pseudoblackleg cultures, or even a pellet of B single vaccine, indicates the absence of any considerable reciprocal immunizing action between the two organisms. These guinea-pigs had shown immunity to several different strains of *Bacillus chauvæi* before the test, so that their subsequent susceptibility to the organisms isolated from the pellets indicates that the organism which we have designated as pseudoblackleg is, in reality, a separate species, and not a separate strain of a polyvalent organism. To what extent blackleg is polyvalent has not been determined, but no indications of a marked polyvalence have been encountered. Guinea-pigs immunized against Kansas strains of blackleg were immune against cultures isolated from vaccines obtained from Europe, and vice-versa.

The fundamental difference between the blackleg and the pseudoblackleg is clearly indicated by experiments with blackleg immune serum, as given in the following. It was found that the blackleg immune serum protected against our pure culture No. 56, and that the same dose of the same serum failed to protect against the pseudoblackleg culture. Many other strains of pure blackleg have been tested against this serum. In every instance the serum has protected the guinea-pigs whether the test dose has been pure culture, or the original blackleg meat. These strains were secured from widely different sources; some of them were isolated from blackleg vaccines obtained from Europe, others from blackleg vaccine manufactured at various places in the United States, and many from naturally occurring cases. The failure to find a single strain derived from such widely different

sources against which the serum would not protect seems to indicate that the polyvalence of the blackleg bacillus is of small importance.

The use of blackleg immune serum in differentiating *Bacillus chauvæi* from closely allied pathogenic anaerobes has been applied by Foth, Meyer,<sup>4</sup> and others. It is considered by these investigators a very useful and reliable test. None of these investigators mentions any noteworthy degree of polyvalency of the blackleg strains with which they have worked.

*Anti-infectious Blackleg Serum.*—Anti-infectious blackleg serum made by the use of pure cultures of the blackleg organism and blackleg muscle juice was found to protect guinea-pigs in 1-c.c. doses against a naturally fatal dose of blackleg, while 1- and 2-c.c. doses of the same serum failed to protect against smaller doses of the pseudoblackleg cultures. The experiments were controlled in such a way as to prove the virulence of the cultures against which the serum was used.

#### SUMMARY

The powder-form vaccines must be given in amounts of from 20 to 150 mg. to kill a guinea-pig.

With 2 exceptions the commercial pellets were not fatal to guinea-pigs in doses of 3 pellets to a guinea-pig. One of these exceptions was an unusually strong serial of a vaccine the virulence of which had previously been low. This pellet produced severe vaccination losses. The other exception was that of B vaccine, which had a M.L.D. of 1 pellet, the strength of which was due to the presence of the pseudoblackleg bacillus.

The pseudoblackleg bacillus differs from *Bacillus chauvæi* in its pathogenicity in rabbits, in the formation of a putrefactive odor, in the size and appearance of the colony in pure culture, in the gas formula, in the blackening of iron-sulfate agar, in the pathogenicity in guinea-pigs highly immune to blackleg, and in the failure of blackleg immune serum to protect guinea-pigs against a lethal dose of pseudoblackleg culture or a B pellet.

<sup>4</sup> Jour. Infect. Dis., 1915, 17, p. 458.

# THE BACTERIOLOGY OF THE URINE IN LOBAR PNEUMONIA \*

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The urine during an attack of lobar pneumonia is decreased in amount and usually contains a trace of albumin and a few granular casts. This so-called febrile albuminuria, or toxic nephritis, which is often associated with other acute infectious diseases, is characterized by certain transitory changes in the kidneys attributed to the action of toxins. Fraenkel and Reiche<sup>1</sup> in an accurate study of these changes found that the cortical layer was almost always exclusively affected, and only in rare instances were there casts in the collecting tubules. In the cortex the secreting parenchyma, the loop of Henle, and the straight tubules alone were involved, and the glomerular capsules contained variable amounts of exudate. The epithelium, except for occasional deposits of pigment, was not degenerated, and there were no recent changes in the medullary connective tissue. In 22 of the 26 cases studied, pneumococci were found in the kidneys, and these observers emphasize the importance of the bacteria in the production of this temporary nephritis. The elimination of the pneumococci through the urine during an attack of pneumonia has often been referred to in the literature, but there is little evidence of a convincing nature to support this view. Should it be proved, however, that pneumococci are commonly found in the urine of pneumonia patients, urine cultures would aid in the diagnosis and the control of the disease. It would be interesting, however, to know whether the pneumococci were in any way changed by their passage through the urinary system. To throw light on these questions bacteriologic examinations of the urine of patients suffering from lobar pneumonia have been made, and the results obtained are here recorded.

## TECHNIC

Urine cultures were made according to the technic described by Dick and Henry.<sup>2</sup> The samples of urine were obtained by catheterization under strict

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<sup>1</sup> Ztschr. f. klin. Med., 1894, 24, p. 230.

<sup>2</sup> Jour. Infect. Dis., 1914, 15, p. 85.

aseptic precautions. A small amount of urine was centrifugated and the sediment examined for casts, leukocytes, bacteria, and other pathologic elements. Cultures were made in human-blood agar plates, the sediment from 10 c.c. of fresh urine being used in varying dilutions. These were incubated for 24 hours at 37 C. All bacteria found that resembled pneumococci or streptococci were studied culturally and biologically, and compared with organisms isolated from the blood or the sputum. Pneumococci could usually be identified by the use of specific sera. In every instance, however, all the usual differential cultural reactions were studied.

Blood and sputum cultures were made according to the technic described in a previous communication.<sup>3</sup>

A bacteriologic examination of the urine and of the sputum or blood was made in 26 cases of lobar pneumonia at various times during the course of the infection. The results are recorded in Table 1. In 10

TABLE 1  
THE URINARY FINDINGS IN 26 CASES OF LOBAR PNEUMONIA

Number of Case	Day of Course	Albumin	Chlorid Retention	Casts	Leukocytes	Micrococci	Pneumococci in Cultures	Other Organisms in Cultures
161	14	—	—	+	—	+	—	Staphylococci
162	12	—	—	—	—	—	—	Staphylococci
163	11	—	—	—	+	—	—	—
164	11	—	—	+	—	—	—	Staphylococci
165	15	—	—	—	+	+	++	—
166	8	+	+	—	—	+	+	—
167	14	+	—	—	+	—	—	—
168	21	—	—	—	—	+	—	Staphylococci
169	4	+	+	+	—	—	—	—
170	10	+	—	+	—	+	+	Staphylococci
171	7	—	—	—	—	+	+	Staphylococci
172	4	—	+	—	—	+	+	—
173	9	—	+	+	—	+	+	—
174	8	+	—	+	—	+	+	—
175	10	—	—	—	+	+	—	—
176	5	—	—	—	—	+	+	Staphylococci
177	9	+	+	—	—	+	+	—
178	21	—	—	—	—	—	—	Streptococcus viridans
180	41	—	—	+	—	+	—	Gram-positive diphtheroid bacilli
181	21	—	—	—	+	+	—	Staphylococci
182	14	—	—	+	—	+	—	—
188	4	—	—	—	—	—	—	—
189	7	—	+	—	—	—	—	—
190	6	+	+	+	—	+	—	Hemolytic streptococcus
191	8	—	+	+	—	+	+	Staphylococci
322	3	+	+	+	—	+	—	Staphylococci

instances, or 38.4%, pneumococci were isolated from the urine. A microscopic examination of the stained smears of the sediment used for cultures revealed the presence of gram-positive diplococci in 18 instances, but in 8 of these the cultures were negative for pneumococci. This discrepancy might be explained by assuming that the organisms in

<sup>3</sup> Mathers: Jour. Infect. Dis., 1915, 17, p. 514.

the smears were staphylococci, or dead pneumococci, for pneumococci do not grow to any appreciable extent in urine. Of the 10 strains of pneumococci isolated from the urine, 5 were found to belong to Group I, 3 to Group II, and 2 to Group IV. These organisms were similar to the strains isolated from the blood or sputum. The biologic and cultural reactions were always the same. Minor variations were noted in the morphology of the organisms isolated from the different sources. These differences, however, are not significant, for the same strain of pneumococcus may vary widely in morphology when observed under different environmental conditions. In 8 cases the cultures were negative. Streptococci were isolated from the urine in 2 instances; they were few in number, and their presence in the urine could be explained on the basis of foci of infection in the mouth, for both cases had numerous alveolar abscesses and marked pyorrhea alveolaris. Other organisms, such as staphylococci and diphtheroid bacilli, were found in several instances.

The urine was examined at various stages of the infection and pneumococci were isolated chiefly at a time just before or just after the crisis. At the onset of the disease or several days after the infection had subsided, the urine cultures were usually negative. The pneumococci were never present in large numbers. Furthermore, the number of pneumococci in the urine did not seem to bear any relation to the other pathologic elements in the sediment. Albumin and granular casts were found during the acute stage of the infection. Altho leukocytes were observed in the stained smears of the sediment, they were few in number, and like the other pathologic elements usually disappeared as quickly as the infection.

#### SUMMARY

During the course of an attack of lobar pneumonia, pneumococci may be excreted in the urine. They appear in the urine usually at a time just before or just after the crisis and do not seem to bear any definite relation to the other pathologic elements in the sediment. The pneumococcus strains isolated from the urine in these experiments are similar to those found in the blood and the sputum.

Pneumococci do not grow in urine to any appreciable extent, but the urine of pneumonia patients should be handled carefully, for it is a possible source of infection in hospitals.

Urinary cultures may be of great value in the diagnosis of pneumonia or of pneumococcal infections in general.

# THE PATHOLOGY OF DERMATITIS VENENATA FROM RHUS DIVERSILOBA \*

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The effect produced by the poisonous sap of poison oak (*Rhus diversiloba* T. & G.) on the human skin is that of an inflammatory process in the cutaneous tissues of several degrees of intensity. For entrance the poison makes use of all possible channels—the sudoriparous and sebaceous ducts, the hair•follicles, and even the surface of the skin itself (see Figs. 1 and 2). Poisoning may occur at any time of the year, but it is most frequent at Berkeley during February, March, and April, as demonstrated by frequency polygons.

The principal lesions of this form of dermatitis have been minutely described by White.<sup>1</sup> The primary lesions are erythema, hyperemic macules, papules, vesicles, and pustules. These may occur as progressive steps in well-defined stages, or they may arise independently and subside without undergoing any further evolutionary change. They may all be present at the same time, or any one may constitute the entire process. The secondary lesions are scale, excoriation, ulcer, and cicatrix. These may take place incident to the decline of the inflammation, or as a result of an accidental interference with its natural course. The subjective symptoms are usually itching and burning in the affected parts. In severe cases this may be intensified to a burning stinging heat, and the torture be so great as to deprive the patient of sleep and require the administration of narcotics.

In this investigation, a microscopical examination of sections of diseased human skin disclosed many leukocytes, among which the mononuclear cells predominated. From this it was thought that leukocytosis might be numbered among the symptoms. The swelling of the lymphatic glands near the affected parts, which is so frequently observed in connection with *Rhus* poisoning, strengthens the probability of its being a phenomenon of adsorption. Investigation showed leukocytosis to be a regular accompaniment of the severer cases from poison oak (see figures). The severity of the leukocytosis seemed to vary directly with the size of the area involved and the severity of the infection. For instance, in a case in which the face, hands, arms, and chest were severely affected a blood count showed 31,600 leukocytes to

\* Received for publication April 1, 1916.

<sup>1</sup> *Dermatitis Venenata*, 1887.

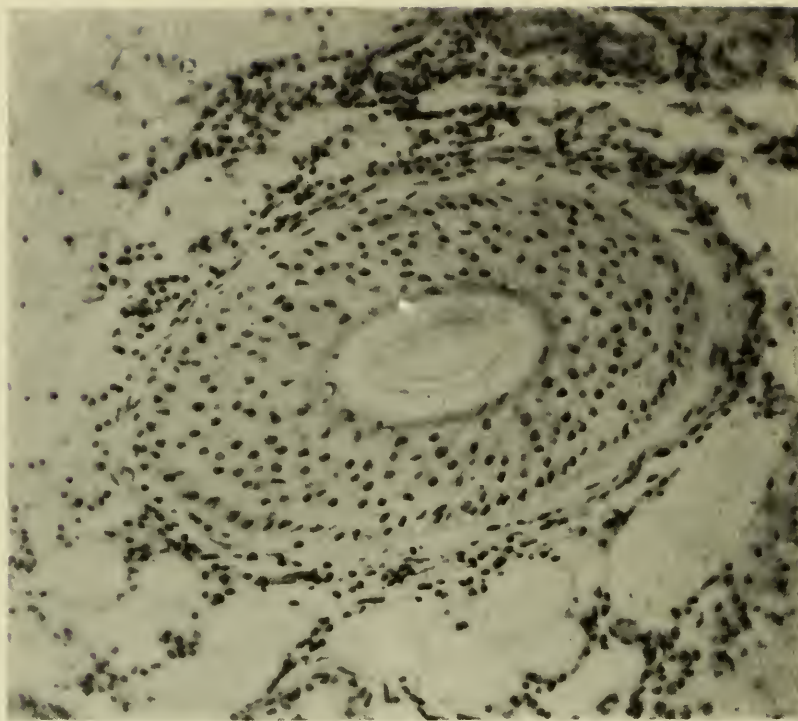


Fig. 1. Section of skin affected by poison oak showing infiltration of leukocytes around a hair follicle.  $\times 190$ .

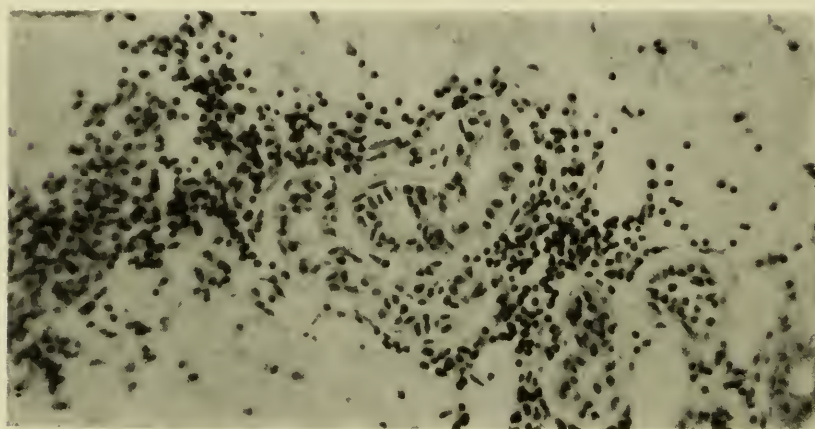


Fig. 2. Section of skin affected by poison oak showing infiltration of leukocytes around a sudoriparous gland.  $\times 190$ .

the cubic millimeter. In a milder case in which only one forearm was affected, 14,000 leukocytes were found to the cubic millimeter of blood. Blood counts frequently indicated the presence of more than 12,000 leukocytes to the cubic millimeter.

Constitutional disturbances may accompany violent cases. The highest body temperature recorded of 45 bed patients was 37.5 C. There is sometimes a slight febrile condition with coated tongue, loss of appetite, and constipation.

As the poison of *Rhus toxicodendron*, a closely related plant, will produce albuminuria in rabbits,<sup>2</sup> it was thought that the poison of *Rhus diversiloba* might cause similar symptoms. With this in view 5 c.c. of the poisonous sap mixed with 15 c.c. of cottonseed oil were introduced per os into a rabbit weighing 3,171 gm. A test for albumin in the urine was found positive 4 hours after the poison was introduced. Hence it was thought albuminuria might occur in severe dermatitis in men. The urine from 28 severe bed cases was examined. All gave negative reactions for sugar. Nineteen, or 68%, were negative with respect to albumin, and 9, or 32%, were positive. One of the patients having albuminuria developed, and recovered from, acute nephritis simultaneously with dermatitis; the rest of the patients showed but faint traces of albumin in the urine.

Tables 1 and 2 describe the case involving a simultaneous acute nephritis.

TABLE 1  
URINE IN WHICH ALBUMIN WAS PRESENT

Sp. Gr.	Color	Appearance	Reaction	Sugar	Albumin
1.012	Straw	Cloudy	Acid	0	Trace
	Light amber	Cloudy	Acid	0	Trace
1.015	Straw	Cloudy	Acid	0	Trace
1.023	Amber	Clear	Acid	0	Trace
1.009	Straw	Clear	Acid	0	Trace
1.032	Amber	Cloudy	Acid	0	Trace
1.018	Amber	Cloudy	Alkaline	0	Trace
			Alkaline	0	Trace
1.008	Amber	Clear	Neutral	0	Trace

The poison in the urine could not be detected in a free condition. Three liters of urine were shaken up with ether, and the ethereal layer separated and concentrated by evaporation. This ethereal extract did not give any chemical reaction for the poison, and when concentrated by evaporation and applied to the skin of a sensitive person, it did not cause dermatitis.

<sup>2</sup> Pfaff: Jour. Exper. Med., 1897, 2, p. 192.

The urine was not abnormal in color, as is sometimes the case when phenol<sup>3</sup> or pyrogallol is externally applied.<sup>4</sup> The urine had no abnormal odor, such as is noticeable upon the inunction of turpentine or drugs of that series.<sup>5</sup>

TABLE 2  
DERMATITIS VENENATA AND ACUTE NEPHRITIS

Time	Temperature, C.	Pulse	Respiration	Analysis of Urine				
				Sp. Gr.	Color	Appearance	Reaction	Albumin
1st day a. m.	36.6	70	16	1.015	Amber	Cloudy	Acid	0.0025 Esbach
2nd day a. m.	36.6	64	18	1.013	Straw	Clear	Neutral	0.006
p. m.	36.9	74	16					
3rd day a. m.	36.4	64	18					
p. m.	36.6	80	16					
4th day a. m.	36.6	68	18	1.015	Amber	Clear	Acid	Fairly large amount
p. m.	36.4	68	18					
5th day a. m.	36.6	84	18	1.013	Amber	Clear	Acid	Fairly large amount
p. m.	36.4	68	18	1.031	Amber	Cloudy	Acid	0.0025
6th day a. m.	36.4	72	18	.....	.....	Cloudy	Acid	Trace
m.	36.5	80	16					
p. m.	36.4	68	18					
7th day a. m.	36.4	68	18	1.036	.....	.....	Acid	Trace
p. m.	37.0	68	16					
8th day a. m.	36.6	68	18					
p. m.	37.0	72	18					
9th day a. m.	36.6	68	18	1.023	Amber	Cloudy	Alkaline	Trace

White blood counts: 1st day, polymorphonuclear 64%, small mononuclear 20%, large mononuclear 6%, eosinophils 10%; 7th day, polymorphonuclear 57%, small mononuclear 35%, large mononuclear 6%, eosinophils 2%. Total count, 13,600.

Heart: Slightly enlarged, action accentuated, premature contraction, otherwise regular, sounds strong; soft systolic at left sternal edge. No edema in ankles. Slight edema in upper and lower eyelids, due at least partly to poison oak. Blood pressure 110. Assythema (asystoles) continues.

Urine: Epithelial casts and cells; no pus.

The seat of the inflammation comprises those parts of the integument which have been in direct contact with the sap of the plant. The parts left unprotected by clothing are generally first affected. The examination of over 1,000 dispensary cases indicates that the parts most usually affected are the backs of the hands, the insides of the forearms, the eyes, ears, and genitalia. Sometimes, however, the eruption is spread by the hands or clothing to other parts of the body. Certain regions are more susceptible than others to the irritant influence; e. g., the face and genitalia, and, indeed, wherever the skin

<sup>3</sup> Köster-Syke: Deutsch. med. Ztg., 1886, 34, p. 381. Browne: Brit. Med. Jour., 1885, 2, p. 692. Dreyfous: Abstracted in L'Union méd. du Canada, 1885, 14, p. 226. Penasse: Jour. de méd. de Paris, 1886, 10, p. 760. Rose: Verhandl. d. Gesellsch. d. Aerzte, 1884. Brun: These de Paris, 1886.

<sup>4</sup> Jarisch: Wien Med. Jahrb., 1878, No. 4. Allen: Jour. Cut. Dis., Incl. Syph., 1886, 4, p. 8.

<sup>5</sup> Brochin: Gaz. d. Hôp., 1879, 52, p. 99. Berenguier: These de Paris, 1874. Garland: Lancet, 1886, 1, p. 1005.

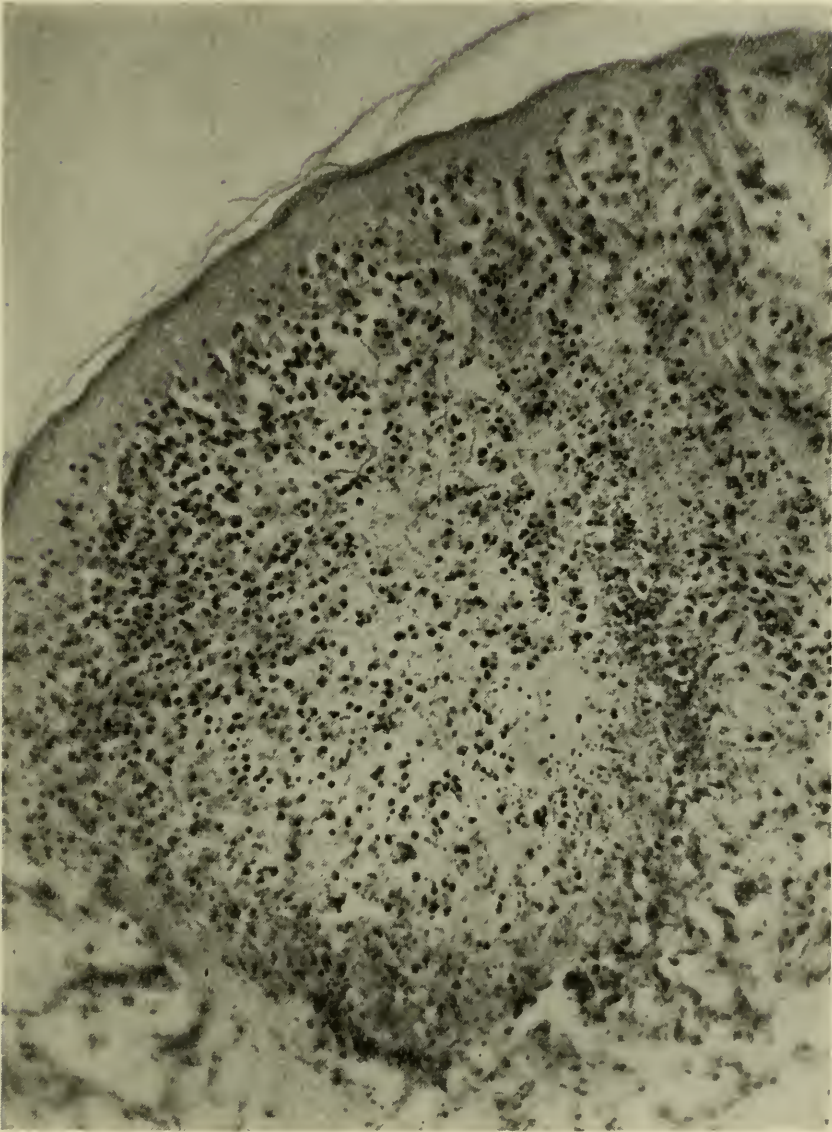


Fig. 3. Section of skin affected by poison oak showing vesicle, infiltration of leukocytes, and slowly diffusible character of the poison.  $\times 190$ .

is thin and delicate. The sticky resinous sap is seldom able to penetrate the thick horny surface of the hands, but it may be spread by them to other parts of the body, e. g., to the genitalia.

The course of the affection is often acute. Within 12 hours after exposure patients frequently break out with a rash. This latent period, or period of incubation, is dependent on the slow diffusibility of the poison into and within the skin, as well as on the predisposition of the individual. The slow diffusibility is evident in microscopical examination (see Figs 3 and 4). The acute symptoms usually subside within from 4 to 6 days. The intensity of the dermatitis depends on the amount of the irritant, and the sensitiveness of the skin; on the author the sap, when well rubbed into the skin of the forearm, produces only

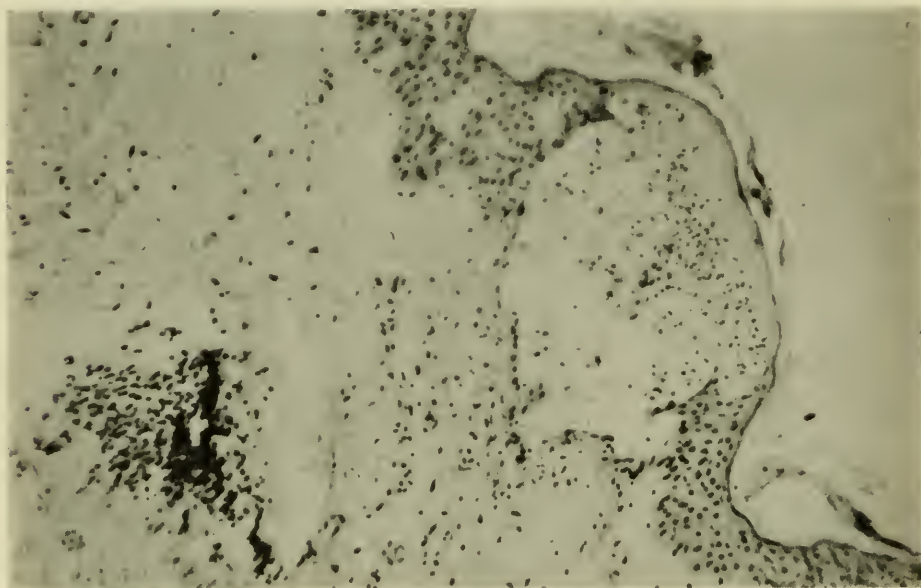


Fig. 4. Section of skin affected by poison oak showing vesicle, infiltration of leukocytes, and slowly diffusible character of the poison.

redness, a slight swelling, and very little pain. The secondary infections of the poison, those caused by its transference from the original place of contact, are always slighter in degree of inflammation, unless the area of the skin is thinner and consequently more sensitive to the poison. This peculiarity is of value in diagnosis. In this connection it should be borne in mind that the exudation from the broken vesicles or papules is nontoxic even when rubbed into the scratched skin of a very susceptible individual. The nontoxicity of the vesicular exudate from this dermatitis is in accordance with the results obtained by White,<sup>1</sup> Van Adelung<sup>6</sup> and Rost and Gilg<sup>7</sup> with *Rhus toxicodendron*.

<sup>6</sup> Arch. Int. Med., 1913, 2, p. 184.

<sup>7</sup> Ber. d. deutsch. pharm. Gesellsch., 1912, 22, p. 296.

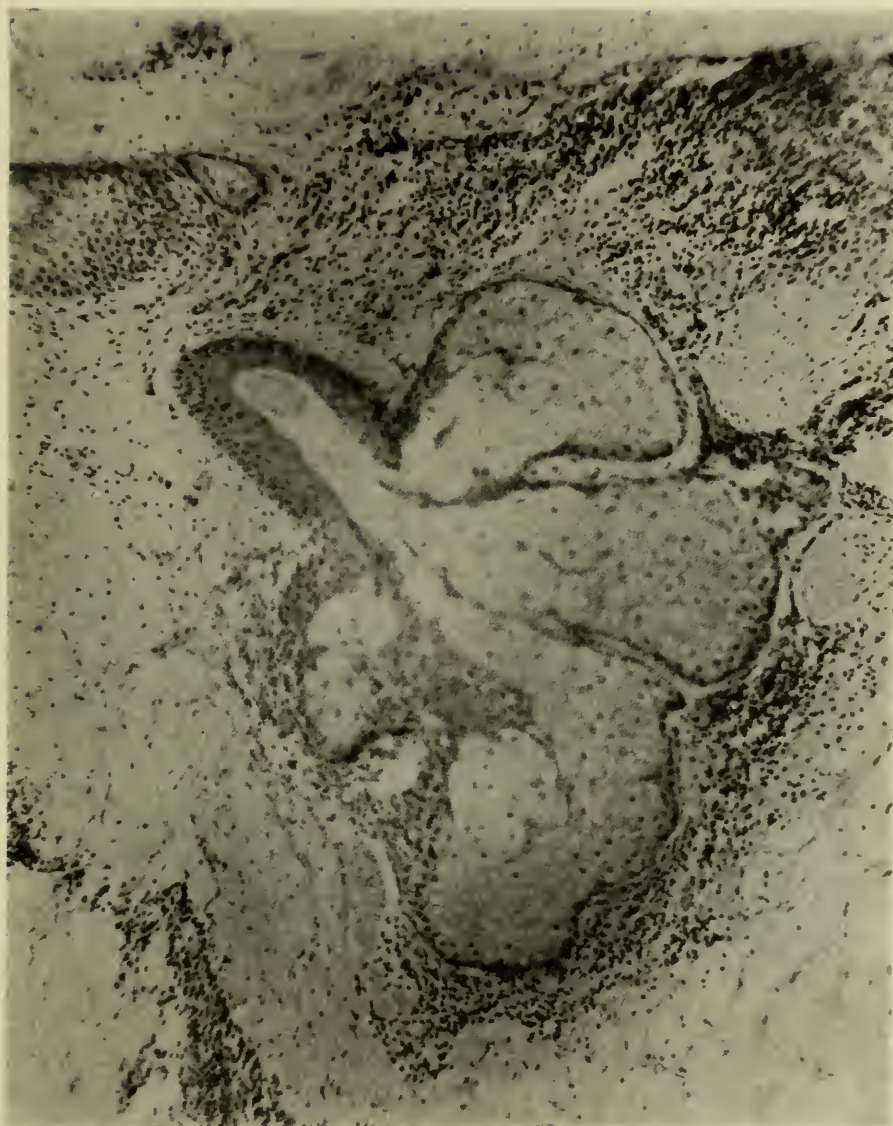


Fig. 5. Section of skin affected by poison oak showing sebaceous gland and infiltration of leukocytes.  $\times 100$ .

It may occasionally be difficult to reach a positive diagnosis at first, as between this type of dermatitis venenata and other forms, including eczema. A history of the onset, occupation, and exposure will be of aid. *Rhus-diversiloba* poisoning frequently begins between the fingers, is markedly acute in character, with a good deal of swelling, and often large vesicles and blebs appear; the latter are rarely seen in eczema. The distribution and configuration of the eruption show certain peculiarities. It often occurs in sharply defined patches, elongated streaks, or irregular shapes as marked out by the original contact with the plant. In distinction from herpes, poison-oak dermatitis does not follow the nerve trunks. Unlike eczema, it seldom attacks the inside of the hands



Fig. 6. Section of skin affected by poison oak showing sebaceous gland and infiltration of leukocytes.  $\times 100$ .

or the scalp. The marked increase in leukocytes, together with the lack of a disproportionate change in polymorphonuclear leukocytes, distinguishes it from the polymorphonuclear character of the dermatitis from turpentine and croton oil, and from the pustular character of dermatitis due to antimony,<sup>8</sup> aconite,<sup>9</sup> and cantharides.<sup>10</sup>

<sup>8</sup> Trousseau: *Treatise on Therapeutics*, 1880, 2, p. 139. Ringer: *Handbook of Therapeutics*, 1897, p. 210. Stille: *Materia Medica and Therapeutics*, 1874, 2, p. 423. Simeons: *Gaz. méd. de Paris*, 1848, p. 192.

<sup>9</sup> Dierbach: Piffard's *Materia Medica and Therapeutics of the Skin*, 1881, p. 12. Farquharson: *Brit. Med. Jour.*, 1879, 1, p. 267. Ringer: *Handbook of Therapeutics*, 1897, p. 397. Stille: *Materia Medica and Therapeutics*, 1874, 2, p. 310.

<sup>10</sup> Trousseau: *Treatise on Therapeutics*, 1880, p. 262. Morrow: Piffard's *Materia Medica and Therapeutics of the Skin*, 1881, p. 38. Dierbach: *Ibid.* Pereira: *Hughes' Manual of Pharmacodynamics*, 1876, p. 244.

1915 - 1916.

men  
women



1914-1915

women  
men



1913 - 1914

women  
men



1912 - 13

men  
women



Frequency polygons of the number of cases of Rhus dermatitis at Berkeley, California.

The acuteness, rather violent characteristics, and distribution of the eruption, together with a history of possible exposure, make up an ordinarily conclusive picture.

#### SUMMARY

The sap of poison oak (*Rhus diversiloba* T. & G.) contains a slowly diffusible skin irritant.

This irritant makes entrance through the sudoriparous and sebaceous ducts, the hair follicles, and through the skin itself.

It produces a dermatitis in many ways similar to many other forms of dermatitis venenata.

Slight constitutional disturbances may take place; a temperature of 37.5 C.; sometimes a febrile condition involving a coated tongue, loss of appetite, and constipation. The urine may have a trace of albumin. One case of acute nephritis is recorded. Sugar has never been noticed.

The seat of inflammation involves that part of the integument which comes in contact with the poison. In over 1,000 cases the backs of the hands, the inside of the forearms, the eyes, ears, and genitalia have been thus involved. The vesicular exudate is nontoxic.

The course of the affection is often acute. It usually reaches its maximum within several days after exposure and may subside within from 4 to 6 days.

Diagnosis may occasionally be difficult. History of the onset, occupation, and exposure is useful. Peculiarities may be noticed in the distribution and configuration of the eruption in distinction from eczema and herpes.

# THE TRANSMISSION OF RHUS POISON FROM PLANT TO PERSON \*

RHUS DIVERSILOBA T. AND G.

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Poison oak (*Rhus diversiloba* T. and G.), like its near relative poison ivy (*Rhus toxicodendron* L.), contains an irritant poison which is capable of affecting the human face so as to cause great discomfort and make recognition impossible. The poison affects different persons differently. Some assert themselves immune, others are poisoned by contact with the plant, and yet others believe that merely to pass the plant in an automobile is sufficient to give rise to an attack of poisoning.

Believers in the toxicity of poison oak may be divided into 2 classes, those who believe the poison to be a gas, and those who consider contact with some part of the plant necessary for infection. These factions obviously cannot be reconciled. The following experiments were carried out to ascertain which opinion is correct.

1.—The poisonous petroleum-ether extract of poison oak was sterilized at 180 F. for 1 hour in an Arnold sterilizer. The extract thus treated was still poisonous, a fact effectually disproving any asserted bacterial nature of the poison. The poison of *Rhus toxicodendron*, its very close relative, was once thought by Burill<sup>1</sup> to be infectious.

2.—The poisonous petroleum-ether extract from 1 pound of leaves was distilled normally until the residue charred. Neither distillate nor residue was poisonous.

3.—The poisonous petroleum-ether extract from 1 pound of leaves was distilled with steam. The distillate was not poisonous, the residue extremely so.

4.—The poisonous petroleum-ether extract from 1 pound of leaves was distilled under 20 millimeters' vacuum until the residue charred. No part of the fractional distillate was poisonous, nor the residue.

5.—One pound of freshly chopped poison oak leaves was placed in a glass alembic. Air at 5 pounds' pressure to the square inch was caused to pass through the leaves and out of the beak. The under side of the wrist was held for 15 minutes at the outlet of the apparatus. No dermatitis developed. One of the leaves thus treated with steam when rubbed on the arm caused dermatitis.

6.—One pound of chopped leaves was treated as described in the foregoing. The outcoming air was bubbled through cottonseed oil for half an hour. The cottonseed oil was not poisonous.

\* Received for publication April 17, 1916.

<sup>1</sup> Am. Mo. Mic. Jour., 1882, 3, p. 192. Proc. Am. Assn. Adv. Sc., 1882, 31, p. 515. Am. Nat., 1883, 17, p. 319. Gard. and For., 1895, 8, p. 368.

7.—Some poison oak leaves were placed in a glass tube and heated until the leaves smoked. This smoke when blown on the wrist caused dermatitis.

8.—The previous experiment was repeated, with the modification of filtering the smoke through glass wool. The glass wool was kept at a temperature of over 100 C. to prevent any possible condensation of the poison. The filtered smoke was not poisonous.

9.—An ethyl acetate solution of the poison had been kept for more than 10 months in an open beaker. More ethyl acetate was added from time to time to replenish that lost by evaporation. This solution was poisonous.

10.—A poison oak leaf was glued to the center of the concave side of a watch glass 6 inches in diameter. A piece of iron window screening 3 inches in diameter was taped in place over the leaf. The watch glass was then taped for 1 hour to the breast of a person very sensitive to the poison. No dermatitis resulted.

The results of the foregoing experiments demonstrate that the poison of *Rhus diversiloba* is nonvolatile, even when mixed with a volatile oil. It may, however, be carried by the particles of soot in smoke.

Of those parts of the plant that might be carried by the wind the pollen, trichomes, and cork cells were investigated.

The pollen was collected by shaking the flowers over a glass funnel to the stem of which a test tube was attached. This pollen was found to be non-toxic when rubbed into the skin. Similar nontoxic results have been obtained with the pollen of *Rhus vernicifera* by Inui,<sup>2</sup> of *Rhus vernix* by Warren,<sup>3</sup> and of *Rhus toxicodendron* L. by Rost and Gilg.<sup>4</sup> Microscopical examination showed 4 resin ducts in the receptacle and pedicel of the male flower, one in each petal. There were no resin ducts in the stamens or their basal filaments. An alcoholic extract of the pollen of *Rhus diversiloba* was nontoxic, nor did it or the pollen assume a dark brown color when treated for 5 minutes with potassium hydroxid, as does the poison.

It is concluded, therefore, that the poisonous principle of *Rhus diversiloba* cannot be transported by the agency of the pollen. The unripe fruit on the female plant is extremely poisonous, but when fully ripe—that is, when the pericarp separates from the waxy endocarp—the drupes are nontoxic.

Schwalbe<sup>5</sup> considered the poison of *Rhus diversiloba* to be excreted from glandular hairs on the surface of the plant. Rost and Gilg<sup>4</sup> have shown that similar hairs are not easily detached from fresh material of *Rhus toxicodendron*. I have found that these hairs, as well as their exudate, are nonpoisonous.

<sup>2</sup> Botan. Centralbl., 1900, 3, p. 352.

<sup>3</sup> Am. Jour. Pharm., 1913, 85, p. 545.

<sup>4</sup> Ber. d. deutsch. pharm. Gesellsch., 1912, 22, p. 296.

<sup>5</sup> Med. Rec., 1903, 63, p. 855. München. med. Wehnschr., 1902, 49, p. 1616.

Two different forms of trichomes have been noticed on the plant, similar to those found by Mobius<sup>6</sup> on *Rhus vernicifera* and by Rost and Gilg on *Rhus toxicodendron*; namely, a unicellular or polycellular needle-shaped hair, and a polycellular club-shaped hair. Morphologically the club-shaped hairs seem to be glandular: 1st, the upper multi-cellular portion is sharply marked off from the basal portion, which resembles a stalk; 2nd, the upper portion has thinner walls than the basal portion; 3rd, they are found mostly on the young rapidly growing organs of the plant, especially the floral region and the leaves, less on the green stem, and hardly at all on the woody portion.

When the green stem, pedicel, or main ribs of the leaf, which are covered with trichomes, are rubbed on sensitive skin no dermatitis results. Care must be taken, however, that the epidermis of the plant is not broken severely enough to cause the resinous sap to exude.

The fresh green leaves were placed in a finger bowl and soaked at room temperature in 95% alcohol for 10 minutes. The leaves had been examined first under a hand lens to make sure that through possible injury no resinous sap was on the surface. When placed in the finger bowl the sap was prevented from running down the pedicel from the cut end into the alcohol. The leaves when taken out of the alcohol, had lost their gloss. The pale yellowish alcoholic solution remaining was concentrated by boiling in an open beaker. It was found to be nontoxic. It was not darkened by potassium hydroxid nor did it respond to other chemical tests for the poison.

These results indicate that neither the plant trichomes nor their exudate is poisonous.

Hubbard<sup>7</sup> advanced the idea that possibly insects carry the poison from plant to person. This theory cannot be denied, but it is doubtful whether many severe cases of *Rhus* dermatitis are caused by the small amount of poison that insects can carry.

The one portion of the plant that is toxic is the resinous sap. This sap rapidly exudes from all parts of the injured plant. When on woolen cloth it preserves its toxic properties for a length of time varying with the atmospheric and temperature conditions to which it is subjected. The sap gradually oxidizes and the poison loses its noxious properties, a black indelible stain being left on the cloth. These stains are often prominently apparent on khaki, linen, etc., after the articles have been laundered. The oxidation is most rapid when the temperature is blood heat and the atmosphere humid. A cold atmosphere delays the oxidation.

The presence of the poisonous sap on clothing accounts for many of the recurrent cases of *Rhus* dermatitis. For example: A lady spent

<sup>6</sup> Abh. Senckenberg, Gesellsch., 1899, 20, p. 210.

<sup>7</sup> Med. Brief, 1904, 32, p. 884.

the night with her sister, whose husband was obliged to be absent on business. The husband had been poisoned about the head and neck with ivy a few days before leaving home. The pillow case on which he slept was not changed, and his sister-in-law, using it, became poisoned about the face and neck in consequence.<sup>8</sup> Other instances of indirect contact with the poison might be cited; for example, handling of cordwood which has some of the poisonous sap on its surface, or handling of tools which have been used in digging up the plant. It is evident that in such cases we deal with results of actual contact through an intermediary agent with the resinous sap of the plant.

#### CONCLUSION

The poison of *Rhus diversiloba* is not carried normally by the wind, for it is neither bacterial nor volatile, and the pollen, the plant hairs, and the cork cells are nontoxic.

Poisoning may occur as the result of direct contact with the unfiltered smoke from the plant, direct contact with the resinous sap, or indirect contact with the resinous sap on clothing, cord wood, tools, etc.

<sup>8</sup> Walker: Med. News, 1891, 59, p. 556.

# THE REFINEMENT AND CONCENTRATION OF ANTITOXINS \*

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The first practical method for the concentration and refinement of diphtheria antitoxin was devised by Gibson.<sup>1</sup> The principle involved in the process was based on experiments carried on by a number of investigators, whose results were on the whole remarkably harmonious. A discussion of earlier work is contained in Gibson's paper. It is generally agreed that the antitoxin in horse blood is contained in the pseudoglobulin fraction.

By the Gibson method the globulins are precipitated by half saturation with ammonium sulfate and the albumins eliminated with the filtrate. Further separation of the globulins is accomplished by dissolving the ammonium-sulfate precipitate in saturated sodium-chlorid solution, and precipitating the antitoxin globulins from this solution with acetic acid. The final precipitate is gathered on hardened filter paper and pressed out, to remove ammonium-sulfate solution as far as possible, then dialysed against running water, and neutralized with sodium carbonate.

Banzhaf later developed a somewhat different method,<sup>2</sup> altho it also was based on the separation of the blood proteins and retention of the pseudoglobulins. The process is as follows:

Citrated plasma is heated to 57 C. for from 12 to 15 hours, and after being cooled, is diluted with half its volume of water. The diluted plasma is mixed with saturated ammonium-sulfate solution in the proportion of 3 parts ammonium-sulfate solution to 7 parts of diluted plasma. The precipitate is gathered on filters and treated in accordance with Gibson's method. The filtrate is mixed with enough saturated ammonium-sulfate solution to bring the saturation to 54%. This second precipitate is gathered on hardened filters, and pressed and dialysed. Neutralization is not necessary. By this method 2 sera are obtained, one of relatively high potency, the other of low.

In 1912 Banzhaf published another paper<sup>3</sup> in which a modification of the previous process is given.

\* Received for publication April 10, 1916.

<sup>1</sup> Jour. Biol. Chem., 1906, 1, p. 161.

<sup>2</sup> Collected Studies from the Research Laboratory, Department of Health, City of New York, 1908-9, 4, p. 230.

<sup>3</sup> Ibid., 1912-13, 7, p. 114.

Citrated plasma is diluted with half its volume of water and enough saturated ammonium-sulfate solution added to make a 30% saturation. The mixture is heated to 60 C. in a water bath, and filtered while hot. The precipitate remaining on the filters is either washed with saturated ammonium-sulfate solution diluted with twice its volume of water, or dissolved in water and ammonium-sulfate solution added to bring the saturation to  $33\frac{1}{3}\%$ . This solution is filtered, the filtrate united with the first filtrate, and enough saturated ammonium-sulfate solution added to bring the saturation to 50%. The resulting precipitate is pressed and dialysed as in the previous method. Only one serum is obtained by this process.

In a report presented by the Committee on Standard Preparation of Diphtheria Antitoxin to the American Public Health Association in September, 1915, the last method of Banzhaf was described with but one modification. This consists of holding the heated plasma-ammonium-sulfate mixture at 60 C. for 15 minutes before filtering.

There are 2 chief economic advantages accruing from the concentration of antitoxin; (1) Blood can be drawn into a solution of some citrate or oxalate and (2) plasma of relatively low value can be utilized. When the blood is drawn into citrate or oxalate solution, coagulation is prevented, the corpuscles settle, and a yield of plasma is obtained which is larger by as much as 50%, than the amount of serum obtained by spontaneous coagulation. The additional expense incurred in refining processes is more than compensated for in this economy.

Other advantages are the somewhat greater stability of the final product, and the fact that a smaller quantity of serum is required for injection, a three- to four-fold concentration being readily obtained. The stability of the product is actually increased, but after some time an opalescence, turbidity, or even a heavy precipitate may form making the serum unsightly, altho its therapeutic value remains unimpaired. Nevertheless physicians frequently object to a turbid serum and manufacturers have found it expedient to warn physicians that turbidity and sediment do not affect the potency of the serum.

If proteins are precipitated in a finished product without reducing the potency, it is clear that there is a possibility of splitting off some protein during the process of refinement without reducing the antitoxic value. Furthermore, the hope that the amount of serum disease following injections of antitoxin would be reduced by using the refined product has been realized in a measure only. On the assumption that serum disease is an anaphylactic reaction it is to be expected that the smaller the number of proteins in a serum, the less the chance of an anaphylactic reaction. Therefore, if the antitoxin globulins can be freed from all traces of other proteins, a high rate of concentration, and a globulin of such purity should be obtained as would greatly

diminish serum disease. The refined products on the market at present are not purified to a degree sufficient to eliminate all nonantitoxic proteins.

Spronck in 1898<sup>4</sup> suggested that the heating of antitoxin to 58 C. for 20 minutes would obviate urticaria. Heating alters some protein, rendering it more precipitable, so that with the elimination of this protein the chances of serum disease are reduced. Heating to 60 C. is also destructive of most bacterial toxins that might accidentally exist in the serum. The aging of serum likewise causes changes in the protein so that serum disease follows its injection less commonly.

It occurred to me that by a combination of methods it might be possible to purify antitoxic serum to a greater degree than heretofore, and during the past year I have succeeded in working out a process which has consistently produced exceptionally high rates of concentration. At the same time the final product is attractive in appearance, green or bluish-green in color, less viscid than some other sera, and water-clear. After 11 months in a syringe one of the first products was still as clear as it was immediately after filtration, altho the color had changed somewhat, the green having passed into a yellowish hue. The quality of the green seems to vary in different sera, but I have observed that with the best results the intensity of the green is pronounced, and I am led to believe that the green color is characteristic of pure globulin solutions.

The process consists of a combination of well-known methods and persistent repetition of certain details until the desired result is obtained. The results are dependable, but in order to obtain uniform products experience and judgment are necessary. The process is briefly as follows:

The first steps are in accordance with Banzhaf's latest method. The citrate or oxalate plasma is diluted with half its volume of water and saturated ammonium-sulfate added in the proportion of 3 parts solution to 7 parts diluted plasma. The mixture is slowly heated in a water bath to 60 C. and held at this temperature for at least 30 minutes. It is then filtered while hot through soft paper filters. After filtration has ceased, the filters with the precipitate collected on them are placed in a suitable vessel, and covered with a measured amount of water. By frequent stirring a fairly homogeneous thick fluid is obtained. To this fluid is added an amount of saturated ammonium-sulfate solution equal to one half the volume of water used. After another thorough stirring the mixture is placed on soft filters and the filtrate mixed with the first filtrate. Finally the filters are filled twice with saturated ammonium-sulfate solution diluted with twice its volume of water. The filtrate contains the last remnants of antitoxin and may be mixed with the previous filtrates. To the

<sup>4</sup> Ann. de l'Inst. Pasteur, 1898, 12, p. 697.

mixed filtrates enough saturated ammonium-sulfate solution is added to bring the saturation to 50%.

It is not necessary to mix the different filtrates. Each one can be precipitated separately to advantage. Since the last filtrates are more highly diluted than the first one they will aid in washing remnants of nonantitoxic proteins from the precipitate which is gathering on the filters. This precipitate is yellowish or yellowish-brown and the filtrate is of similar color. After filtration has ceased, the filters with the precipitates are stirred in a measured amount of water; after solution the fluid is strained through cheese cloth and the remaining paper pulp pressed out. The pressed pulp is again mixed with a measured amount of water, strained and pressed, and this process repeated a third time. All globulins are washed out of the paper by this method.

The strained fluids are mixed and a volume of saturated ammonium-sulfate solution equal to the total amount of water used for solution is added. The precipitate is gathered again on soft filters. The precipitate and filtrate are now much lighter in color than after the first precipitation.

The precipitate should be dissolved and precipitated a second, or even a third, time until the precipitate is bluish-green and the filtrate colorless. Nonantitoxic proteins are present as long as the filtrate is colored yellow or yellowish-brown. The precipitate can now be pressed out and dialysed. In most cases better results are obtained when heat is applied a second time. To this end the precipitate is dissolved in a measured amount of water, the paper pulp pressed out as before and the strained fluid measured. The fluid is of course of a larger volume than the amount of water added for solution, because of the volume occupied by the precipitate and the presence of some 50% ammonium-sulfate solution in the moist precipitate. It may be assumed that the difference in volume is half the saturated ammonium-sulfate solution, altho it is really somewhat short of the assumed amount, and on this basis enough saturated ammonium-sulfate solution is added to make a 30% saturation. A very slight precipitate will form if enough ammonium sulfate is present. If this precipitate does not appear more ammonium-sulfate solution should be added until a slight cloudiness is produced. This mixture is then heated to 60 C. again, and held at this temperature for 15 minutes. It is then filtered while hot, and the precipitate washed out as before. The mixed filtrates are brought to a 50% saturation with ammonium-sulfate solution and the precipitate gathered on hard filters. The color of the precipitate is now bluish-green, and after the ammonium-sulfate solution has been pressed out, dialysis can be commenced. The pressed precipitate is intensely bluish-green, resembling a substance containing copper. By the second heating a precipitate is formed which is not soluble in 30% ammonium-sulfate solution and which perhaps consists of a globulin which has been split off.

The resulting globulin solution is generally satisfactory. It may not be quite water-clear, but is green and passes readily through a Berkefeld filter, as it is not very viscous. By this method I have obtained sera of 800, 900, and in one instance 1000 units from plasma which tested less than 200 units, and probably but little more than 100 units.

Frequently the serum at this stage is turbid. This seems to occur chiefly when the blood is obtained from old horses or from a horse

that has been bled to death. A serum of this kind can be clarified completely by the following method:

It is diluted with twice its volume of water and enough saturated ammonium-sulfate solution to make a 7 to 3 saturation is added. This mixture is heated to 60 C. and filtered while hot. The precipitate which remains on the filters is dissolved in water and saturated ammonium-sulfate added to make a one-third saturation. This mixture is filtered and the filtrates united as in the beginning of the process. The globulins are precipitated by addition of saturated ammonium-sulfate solution to a 50% saturation. The precipitate when gathered on the hard filters is intensely bluish-green and after dialysis the solution is always perfectly clear. There is a loss of about one-third of the volume and a corresponding increase in potency. By this method of double concentration I have obtained sera of from 1,200 to 1,400 units' potency from plasma testing less than 200 units, and in one instance a 2,000 unit serum from plasma testing less than 300 units.

The process seems of course rather laborious. Most of the work, however, can be done by inexpensive helpers. The expense of ammonium sulfate is somewhat greater than in the case of the former method, but this expense can be reduced by using the last colorless filtrate over again as containing a 50% saturated ammonium-sulfate solution. It can be used whenever a 30 or 33% saturation is required. A large number of soft filters are used, but relatively few hard filters are required. The cost of filters is therefore not greatly increased since hard filters are much more expensive than soft ones. It is not essential to heat the globulin solution more than once during the first concentration if a second concentration is to be carried out.

The advantages gained appear quite sufficient to offset the extra expense, for a relatively high potency serum with 18 to 20% solids can be obtained from a plasma which otherwise would be valueless, and the stability of the serum is also of considerable economic value.

Sera prepared by the described method have been used in the Durand Hospital of the Memorial Institute for Infectious Diseases in Chicago. Dr. George H. Weaver has made the following observations on the occurrence of serum disease after injections of a serum prepared by the new process as compared with serum prepared by the Banzhaf method: "Serum No. 446, concentrated according to Banzhaf's method, was used in 21 cases. Reactions were observed in 10 cases, or 47.6 percent. Of these reactions two were severe, four moderate, and four mild. Serum No. 519, concentrated according to Banzhaf's method, was used in 31 cases. Reactions were observed in 12 cases, or 38.7 percent. Of these seven were severe, two moderate and three mild. Serum No. 543, concentrated according to the new method, was

used in 40 cases. There were but three reactions, or 7.5 percent. Of these one was severe and two moderate. The serum was used in a number of additional cases, but since these had been treated with antitoxin before being taken to the hospital they had to be excluded."

There is little doubt about the feasibility of concentrating tetanus antitoxin according to the same principle. I have myself concentrated hog-cholera serum successfully. The serum used was sent to me for experimental purposes by Dr. C. H. Stange of Iowa State College at Ames. The final product was sterile, and water-clear with a slightly pinkish color due to remnants of hemoglobin which were impossible to exclude, tho if hog's blood were drawn into a citrate or oxalate solution a perfect product could be obtained. In this case 7 c.c. of the refined product protected, while of the original serum from 20 to 30 c.c. were required. This work bears out a statement made by Eberson<sup>5</sup> that the antibodies of hog-cholera serum are contained in the pseudoglobulin fraction of hog's blood.

The work reported in this paper demonstrates 2 facts: first, antitoxic sera concentrated by the Banzhaf method contain, besides pseudoglobulins, varying amounts of other blood proteins which are not readily eliminated. What these proteins are I have not determined, but the results of my work suggest that precipitation of protein in serum when it ages is due to the presence of nonantitoxic proteins and not to true antitoxic globulins. Moreover, there is support for the belief that serum disease is largely due to the presence of these nonantitoxic proteins. Therefore, the amount of serum disease is reduced in proportion to the completeness of the elimination of nonantitoxic proteins.

Second, it has been demonstrated that pseudoglobulins may be split into fractions and that some of these do not contain antitoxin while others contain it in highly concentrated form. The possibility suggests itself that by repeated heating of antitoxic serum with 30% ammonium-sulfate solution the antitoxin may be condensed into a very small fraction of pseudoglobulin. If this were successful considerable light might be thrown on the condition in which antibodies exist in the blood. The high rate of concentration resulting from my method is due of course simply to the elimination of nonantitoxic proteins, which otherwise would require a greater amount of water for solution, unless the solids in the final product are excessively high.

<sup>5</sup> Jour. Infect. Dis., 1915, 17, p. 339.

## CONCLUSION

A serum of attractive appearance can be produced. It is water-clear for at least 1 year, probably for a much longer period. No turbidity develops, nor does a sediment form during this time.

A higher concentration can be obtained than by former methods, because nonantitoxic proteins are eliminated. The solids of the product obtained by the new method need not be higher than from 18 to 20%.

The consistency of the serum enables it to flow readily through Berkefeld filters, a pressure of from 15 to 20 pounds being usually sufficient to maintain a constant flow when "v" or "n" density filters are used. Furthermore, it is easily discharged through a hypodermic needle.

The antitoxic globulins realized by the new method are easily soluble and dialysis is consequently rapid.

Plasma of 100 and 200 units can be used to advantage, thus being offset the additional expense of production.

Nonantitoxic proteins are eliminated in large measure and serum disease, as far as present observations go, is greatly reduced.

# THE CYTOLOGY OF THE EXUDATE IN THE EARLY STAGES OF EXPERIMENTAL PNEUMONIA \*

PLATES 15 AND 16

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On examination of sections from the consolidated lungs of pneumonia patients dying in the first few days of the disease it is readily appreciated that the cellular characteristics of the exudate in the early stages are not the same as in the almost exclusively polymorphonuclear exudate of the later stages. The early exudate is predominantly mononuclear in character, and is to be differentiated from that of the very late stages when again mononuclear cells are frequently numerous. In the later stages the mononuclear cells present are plasma cells and desquamated alveolar epithelial cells. The mononuclears seen in the early exudate are not, however, so readily classified. Several distinct types must be recognized (Fig. 1), and each type is constant in its occurrence. It has been our aim to identify some of these cells in the pneumonic processes experimentally induced in rabbits, and in the processes early in 3 cases of human pneumonia.

The pneumonic process was induced in each rabbit by intrabronchial injection of 8 c.c. of an 18-hour neutral-broth culture of the organism after the method of Meltzer and Lamar.<sup>1</sup> The organisms injected were strains from *Pneumococcus* Group I and Group IV, an attenuated strain of pneumococcus of undetermined group, a hemolytic streptococcus, and a streptococcus isolated from the mouth of a normal individual. The exudate in reaction to intrabronchial injection of 33% egg yolk in neutral broth was also studied.

The lesions were examined at periods varying from 8 to 36 hours. In some cases the gross distribution of the process was fairly regular, embracing an entire lobe or all the lobes of one side, tho it was never entirely homogeneous as in human lobar pneumonia; in other cases the distribution was patchy. The even distribution was commonly encountered after pneumococcus injection, and the patchy after the injection of a streptococcus, but this was not constant. In a number of the rabbits the alveolar walls were infiltrated, the infiltrating cells being of the same character as those in the exudate (Fig. 1). The cytology

\* Received for publication April 18, 1916.

<sup>1</sup> Jour. Exper. Med., 1912, 15, p. 133.

of the early exudates in the three human cases of pneumococcal pneumonia, coming to autopsy on the 3rd, 4th, and 5th days respectively, was the same as that of the experimental pneumonia in rabbits (Fig. 4).

Except for slight variations in the relative proportions in which the cells were present, depending to some extent on the organisms, but more particularly on the age of the exudate, the cytology was the same in all the preparations. Typical small lymphocytes of the blood were always present in small numbers and were easily identified. Even in the very early exudate some polymorphonuclears were constantly seen, perhaps only one to an alveolus in some places, but to the exclusion of all other types in other alveoli in the same section. A few very large mononuclear cells also were frequently encountered which, by reason of their morphology and staining properties, could be definitely classified as epithelial cells in various stages of degeneration. In addition to these there were other mononuclears which were the predominating mononuclear cells, and except in those alveoli where the polymorphonuclears outnumbered them, they were the predominating cells of the exudate. These cells, always much larger than the polymorphonuclears, yet smaller than the epithelial cells, fell into 2 groups, depending on whether or not they contained an oxydase ferment as demonstrated by the indophenolblue reaction<sup>2</sup> (Fig. 2). Other differential criteria were indefinite except that those containing an oxydase ferment commonly had a slightly or markedly indented nucleus with only a fair amount of chromatin and abundant homogeneous protoplasm that occasionally showed faintly a fine granulation, whereas those cells that did not contain an oxydase ferment had, as a rule, a round or oval nucleus often showing one or two flat surfaces and sometimes staining deeply, and abundant homogeneous protoplasm that never showed evidence of granulation. In form the typical cells of the first group strongly suggested the so-called transitional cells of the blood and were so considered by Pratt<sup>3</sup> in 1900. Their content of oxydase ferment makes this seem likely if myelocytes are not considered. Both myelocytes and so-called transitional cells give the oxydase reaction, and as Naegeli has pointed out<sup>4</sup> the differentiation between them in tissue sections is sometimes difficult. Certainly a few cells resembling myelocytes were to be seen. Myelocytes probably were present in small numbers, but, altho they must be considered, the morphology of most of the cells under discussion was not that of myelocytes and it

<sup>2</sup> Evans: Proc. New York Path. Soc., 1915, 15, p. 143.

<sup>3</sup> Johns Hopkins Hosp. Rep., 1900, 9, p. 265.

<sup>4</sup> Blutkrankheiten und Blutdiagnostik 1912, p. 193.

seems quite certain that they belonged to the so-called transitional-cell group. The other cells, those that did not give the oxydase reaction, were probably lymphoid in origin, and many of them resembled very much the pictures of polyblasts appearing in Maximow's articles.<sup>5</sup> They were not reticulo-endothelial cells, for tho such cells, vitally staining by carmin, are occasionally seen normally in the alveolar walls, in pneumonia in rabbits heavily stained by carmin these carmin cells were not seen taking part in the formation of the exudate. That they are closely related to the blood elements is further borne out by the fact that in pneumonia induced in rabbits poisoned by benzol,<sup>6</sup> whereby the leukocytes and leukopoietic tissue were destroyed, cells were seen in the exudate (Fig. 3). No plasma cells were seen in any of these preparations.

#### CONCLUSION

In the early exudate of pneumonia, altho many polymorphonuclears may be present, in many alveoli the cytology of the exudate is predominantly mononuclear in character. The mononuclear cells may be classified as follows: (1) a few typical small lymphocytes, (2) a few desquamated alveolar-wall epithelial cells, (3) relatively many oxydase-containing large mononuclears of the blood, belonging to the so-called transitional-cell group of Naegeli, and (4) almost as many non-oxydase-containing large mononuclears of the blood or closely related forms.

#### EXPLANATION OF PLATES

- A = small lymphocytes.
- B = polymorphonuclears.
- C = desquamated epithelial cells.
- D = oxydase-containing large mononuclears.
- E = non-oxydase-containing large mononuclears.

FIG. 1. Section of consolidated rabbit lung in which pneumonia has been induced by intrabronchial injection of *Streptococcus hemolyticus*. Animal killed 14 hours after inoculation. The cells infiltrating the alveolar walls are essentially the same as those in the alveolus.

FIG. 2. The indophenolblue reaction on a section of consolidated rabbit lung after pneumococcus injection. The nuclei are counterstained by 5% aqueous pyronin.

FIG. 3. Section of consolidated rabbit lung in which pneumococcal pneumonia has been induced after destruction of the leukopoietic tissue by administration of benzol.

FIG. 4. Section of consolidated human lung from a pneumococcal pneumonia, patient dying on the third day of the disease. This also represents accurately the condition in induced pneumococcal pneumonia in rabbits.

<sup>5</sup> Beitr. z. path. Anat. u. z. allg. Path., 1903, 34, p. 153; 1904, 35, p. 93; 1905, 38, p. 301.

<sup>6</sup> Selling: Ibid., 1911, 51, p. 576.

PLATE 1

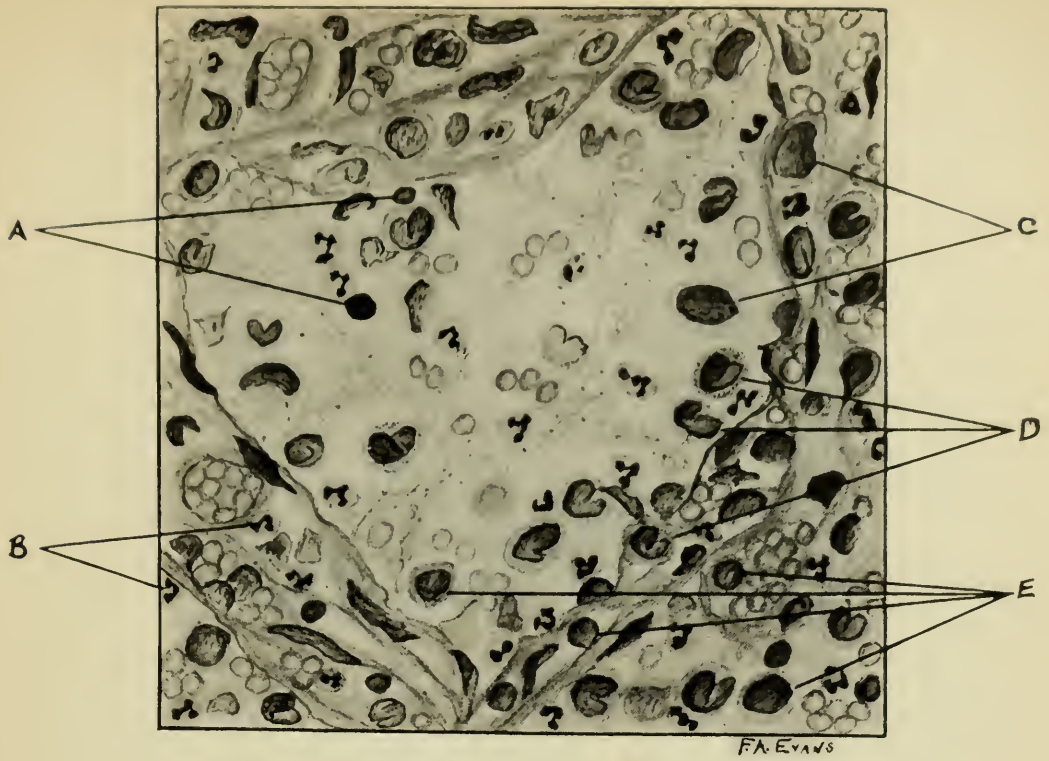


Figure 1

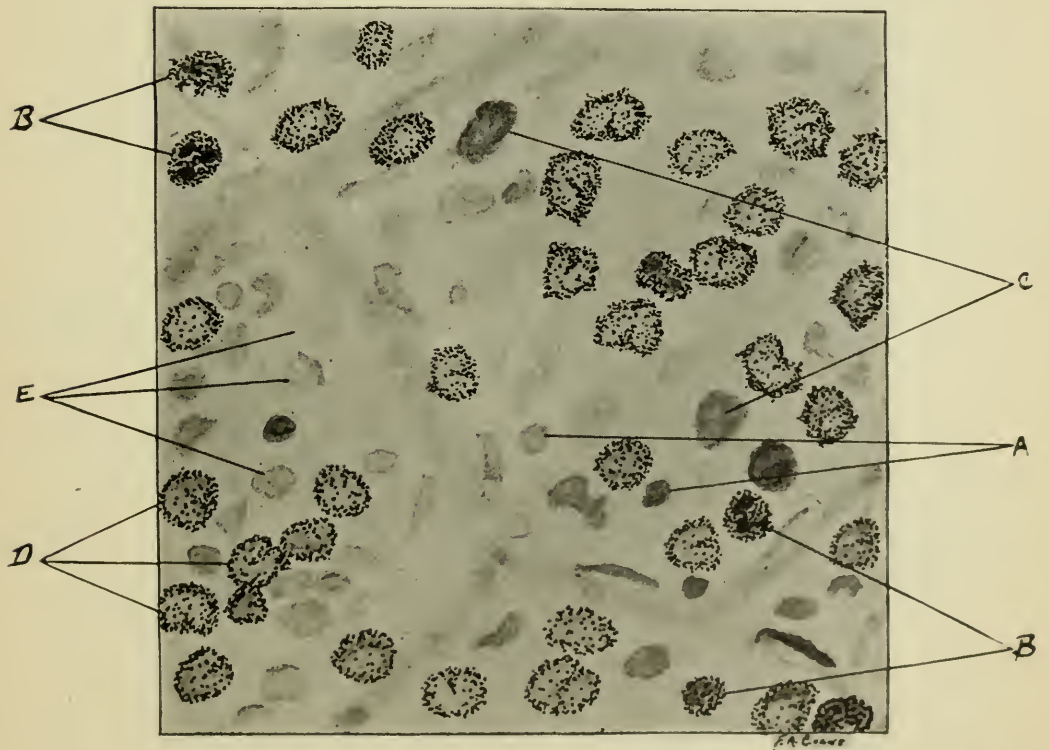


Figure 2



PLATE 2

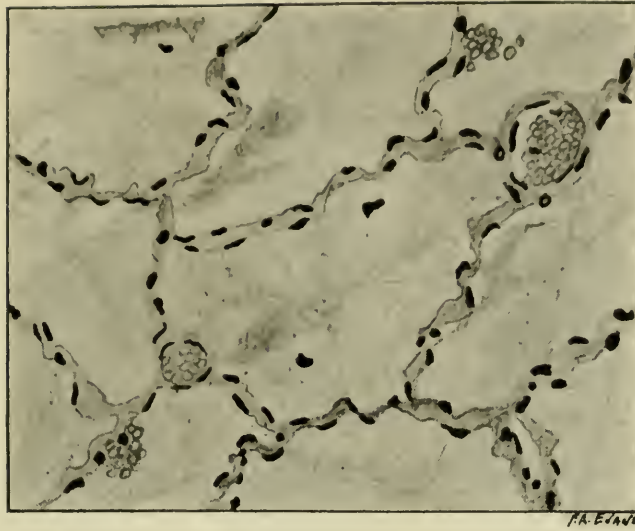


Figure 3

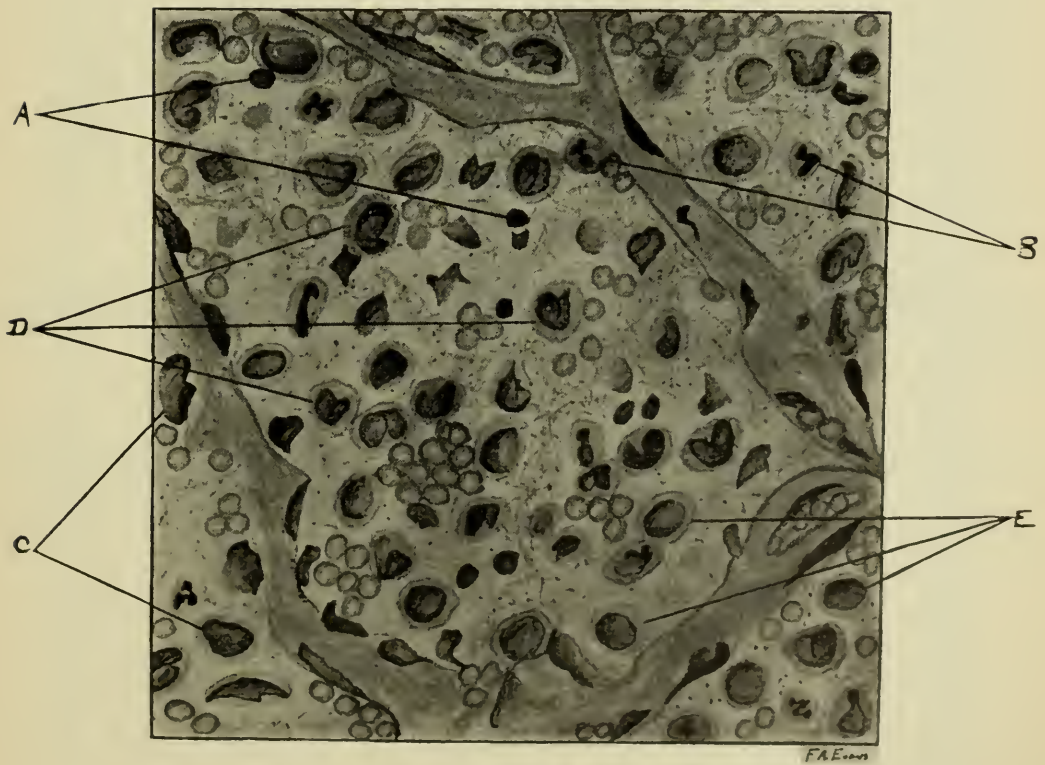


Figure 4

# AGGLUTININS IN HOG-CHOLERA IMMUNE SERUM FOR BACILLUS SUIPESTIFER \*

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The eventual value of hog-cholera immune serum for the treatment of chronic hog cholera makes it seem of advantage to know something concerning the quantity of antibodies against *Bacillus suipestifer* in the commercial hog-cholera immune serum. So far as agglutinins are concerned, data were obtained by the following tests.

Blood was drawn from the tail or from the jugular vein; it was at once defibrinated and centrifugated. A 10% dilution of the serum in natural salt solution was made and from this further dilutions as follows:

Tube	Salt Solution, c.c.	Serum, c.c.	Bacterial Emulsion, c.c.	Dilution
1	...	0.5 (10%)	0.5	1:20
2	0.5	0.5 (10%)	0.5	1:40
3	0.5	0.5 from Tube 2	0.5	1:80
4	0.5	0.5 from Tube 3	0.5	1:160
5	0.5	0.5 from Tube 4	0.5	1:320
6	0.5	0.5 from Tube 5	0.5	1:640
7	0.5	0.5 from Tube 6	0.5	1:1280
8	0.5	0.5 from Tube 7	0.5	1:2560
9*	0.5	0.5 from Tube 8	0.5	1:5120

\* 0.5 c.c. is removed from Tube 9.

The bacterial emulsion was made by washing off agar cultures of *B. suipestifer*, 24 hours old. The strain was sent to us from the New York Museum of Natural History.

TABLE 1  
THE AGGLUTININS IN VIRUS PIGS (AVERAGE WEIGHT 75 LB.)

Pig	Date of Infection	Date of Bleeding	Agglutination Titer
1	April 15	April 26	—
2	Contact infection	April 28	1:40
3	Contact infection	April 28	—
4	April 21	April 28	1:40
5	April 21	April 28	—
6	April 21	April 28	—
7	April 21	April 28	1:20
8	April 21	April 28	—

\* Received for publication April 20, 1916.

TABLE 2

THE AGGLUTININS IN HYPERIMMUNE PIGS (AVERAGE WEIGHT 250 LB.)

Pig	Date of Hyper-immunization	Date of Rehyper-immunization	Date of Bleeding	Agglutination Titer
1	February 4	March 10	April 6	1:160
2	February 6	March 12	April 6	1:1280
3	February 6	March 12	April 6	1:320
4	February 8	March 13	April 6	1:40
5	February 9	March 13	April 6	1:640
6	February 8	March 12	April 6	1:80
7	February 20-25	March 22	April 8	—
8	February 20-25	March 22	April 8	1:160
9	February 25	March 22	April 8	1:160
10	February 25	March 22	April 8	1:320
11	February 25	March 22	April 8	1:1280
12	February 25	March 22	April 8	1:320
13	February 25	March 22	April 8	1:20
14	February 25	March 22	April 8	1:80
15	February 15	March 24	April 9	1:80
16	February 15	March 23	April 9	—
17	February 15	March 23	April 9	1:640
18	February 15	March 23	April 9	1:40
19	February 15	March 23	April 9	—
20	February 20	March 24	April 9	1:20
21	February 20	March 24	April 9	1:1280
22	February 20	March 24	April 9	1:20
23	February 15	March 25	April 12	1:40
24	February 16	March 25	April 12	1:1280
25	February 16	March 25	April 12	—
26	February 16	March 25	April 12	1:20
27	February 16	March 25	April 12	1:20
28	February 20	March 25	April 12	1:80
29	February 23	March 26	April 12	1:20
30	February 23	March 26	April 12	1:160
31	February 8	March 22	April 14	1:1280
32	February 15	March 23	April 14	—
33	February 15	March 23	April 14	1:2560
34	February 15	March 23	April 14	1:40
35	February 17	March 22	April 14	1:80
36	February 17	March 22	April 14	1:160
37	February 17	March 22	April 14	1:80
38	February 17	March 22	April 14	1:40
39	February 17	March 30	April 16	1:160
40	February 17	March 30	April 16	1:320
41	February 17	March 29	April 16	1:1280
42	February 17	March 29	April 16	1:80
43	February 17	March 29	April 16	1:320
44	February 17	March 29	April 16	1:320
45	February 28	March 30	April 16	1:5120
46	February 28	March 30	April 16	1:1280
47	March 1	March 31	April 17	1:640
48	March 1	March 31	April 17	1:1280
49	March 1	March 31	April 17	1:160
50	March 1	March 31	April 17	1:5120
51	March 1	March 31	April 17	1:640
52	March 1	March 31	April 17	1:6000
53	March 1	March 31	April 17	1:640
54	March 3	April 3	April 17	1:160
55	February 17	April 1	April 19	1:640
56	February 17	April 1	April 19	1:6000
57	February 17	April 1	April 19	1:10000
58	March 1	April 1	April 19	1:2560
59	March 3	April 3	April 19	1:80
60	March 2	April 2	April 19	1:160
61	March 2	April 2	April 19	1:320
62	March 2	April 2	April 19	1:160
63	February 10	April 10	April 20	1:80
64	March 6	April 10	April 20	1:320
65	March 3	April 3	April 20	1:320
66	March 3	April 3	April 20	1:640
67	March 3	April 3	April 20	1:20
68	March 3	April 3	April 20	1:320
69	March 3	April 3	April 20	1:160

TABLE 2—Continued  
THE AGGLUTININS IN HYPERIMMUNE PIGS (AVERAGE WEIGHT 250 LB.)

Pig	Date of Hyper-immunization	Date of Rehyper-immunization	Date of Bleeding	Agglutination Titer
70	March 3	April 3	April 20	1:100
71	February 17	March 30	April 23	1:320
72	February 17	March 29	April 23	1:1280
73	March 1	March 31	April 23	1:640
74	March 1	March 31	April 23	1:1280
75	March 1	March 31	April 23	1:80
76	March 1	March 31	April 23	1:5120
77	March 1	March 31	April 23	1:320
78	March 1	March 31	April 23	1:2560
79	February 17	April 15	April 24	1:2560
80	February 17	April 15	April 24	—
81	February 19	April 15	April 24	1:20
82	March 6	April 14	April 24	1:320
83	March 6	April 14	April 24	1:640
84	March 6	April 15	April 24	1:2560
85	March 6	April 15	April 24	1:20
86	March 6	April 15	April 24	1:2560
87	February 17	April 16	April 26	1:320
88	February 17	April 16	April 26	1:40
89	February 17	April 16	April 26	—
90	February 17	April 17	April 26	1:80
91	February 19	April 17	April 26	1:2560
92	February 19	April 17	April 26	1:160
93	February 19	April 16	April 26	1:20
94	February 17	April 7	April 27	1:5120
95	March 2	April 2	April 27	1:160
96	February 17	April 1	April 27	1:640
97	February 17	April 2	April 27	1:640
98	February 17	April 1	April 27	1:1280
99	February 17	April 1	April 27	1:80
100	February 17	April 1	April 27	1:320

Of 8 normal pigs, 5 showed no agglutination of *B. suispestifer*, while 2 gave agglutination in 1:10, and 1 in 1:40.

As Tables 1 and 2 indicate, the high titers were present only in the sera of hyperimmune pigs. The agglutinins were produced not as a result of acute cholera, but as a result of the presence of *B. suispestifer* in the large amount of virus blood injected (5 c.c. to each pound of the pig). In the average the titer was 1:1000, or exactly 1:999.5. Twenty-one pigs had a titer under 80, and in these cases I assume that no considerable quantity of *B. suispestifer* had been injected. Often the blood of one virus pig was used for the hyperimmunization of one immune pig. Mixtures of different blood specimens were however usual. In our case it could be rightly supposed that in 21% of the hyperimmunizations the blood was practically free from *B. suispestifer*. This percentage, representing the absence of *B. suispestifer* in 100 cases of hog cholera, is of course too low and this circumstance is most likely due to mixture of the viruses and to the persistence of agglutinins from the first hyperimmunization. However, I have reason to believe that here in Ames the percentage of cases of hog cholera mixed with secondary infections of the paratyphosis-B. type is above 44.6%

(Uhlenhuth's number). Eberson<sup>1</sup> found under the same conditions 28 of 55 virus pigs infected with *B. paratyphosus* B.

To demonstrate any relation that might exist between the strength of the hyperimmune serum and the agglutinating titer for *B. suispestifer*, the following experiment was made:

From 8 immune pigs ready to be hyperimmunized, blood was taken and the agglutinating titer for *B. suispestifer* found. The titers were as follows:

Pig	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Titer
1	+++	+++	++	+	—	—	—	1:80
2	+++	+++	+	—	—	—	—	1:40
3	++	+	—	—	—	—	—	1:20
4	++	+	—	—	—	—	—	1:20
5	+++	+++	+++	+++	++	+	—	1:320
6	++	++	+	—	—	—	—	1:40
7	+++	++	+	—	—	—	—	1:40
8	++	+	—	—	—	—	—	1:20

The pigs, which all weighed near 200 pounds, were at the same time hyperimmunized with 5 c.c. of virus blood to 1 pound of body weight. The virus blood was taken from several pigs and carefully mixed, so that each pig would get the same amount of virus and bacteria. After 10 days blood was drawn again from the hyperimmunized pigs and the agglutinating power tested. Three pigs were accidentally lost. The titers of the 5 left were as follows:

Pig	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:6000	1:8000	1:10000
1	+++	++	+	—	—	—	—	—	—	—	—	—
4	++	+	—	—	—	—	—	—	—	—	—	—
5	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	—
7	+++	+++	+++	+++	++	—	—	—	—	—	—	—
8	+++	++	—	—	—	—	—	—	—	—	—	—

Then the strength of these sera as hog-cholera immune sera was tested. Eight young pigs of about equal weight were selected for each serum and pairs of them injected severally with different amounts of serum and 2 c.c. virus. The virus was previously tested out to determine its virulence. The results are given in Table 3.

All the test pigs showed febrile reactions. The cause of the deaths was ascertained by postmortem examinations.

Serum 7 and Serum 8 were the strongest so far as protective power against hog-cholera virus is concerned, a fact which is not in accordance with the result of the agglutination tests.

The experiment was not carried out further, because conclusions were reached from other experiments (report of which will be published in the near future) indicating the improbability of a connection,

<sup>1</sup> Jour. Infect. Dis., 1915, 17, p. 331.

TABLE 3  
PROTECTIVE POWER OF HYPERIMMUNE SERA AGAINST HOG CHOLERA

Test Pigs	Amount of Serum in c.c.	Result
Serum 1		
1	20	—
2	20	—
3	15	—
4	15	Died after 16 days. H. C.
5	10	—
6	10	—
7	5	Died after 18 days. H. C.
8	5	Died after 10 days. H. C.
Serum 4		
9	20	—
10	20	—
11	15	—
12	15	—
13	10	—
14	10	—
15	5	Died after 13 days. H. C.
16	5	Died after 10 days. H. C.
Serum 5		
17	20	—
18	20	—
19	15	—
20	15	—
21	10	—
22	10	—
23	5	Died after 15 days. H. C.
24	5	Died after 14 days. H. C.
Serum 7		
25	20	—
26	20	—
27	15	—
28	15	—
29	10	—
30	10	—
31	5	—
32	5	—
Serum 8		
33	20	—
34	20	—
35	15	—
36	15	—
37	10	—
38	10	—
39	5	—
40	5	—

and because the negative outcome had a much higher determining value than an eventual positive one. Pfeiffer and Kolle and Dieudonné<sup>2</sup> have shown that there is no connection between immunity and agglutinins;

<sup>2</sup> Cited by Paltauf, Kolle und Wassermann's Handb. d. pathogen. Mikro-organismen, 1913, 2, p. 483.

the same authors and many others have demonstrated that the amount of bactericidal immune bodies is not analogous to the amount of agglutinins in an immune serum against the same antigen.

#### SUMMARY

The paper presents tabulations of the agglutinins for *B. suipestifer* in 100 hog-cholera immune sera, in 8 sera of normal pigs, and in 8 sera of virus pigs, also the report of an experiment to ascertain the connection between the agglutinin titer for *B. suipestifer* and the amount of virus antibodies in a hyperimmune serum.

# COLLOIDAL CHEMISTRY AND IMMUNOLOGY \*

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In every immunologic research in vivo as in vitro, we shall always have to remember the fact that all the substances that we are treating (and considering) either are themselves colloidal substances, or are closely and inseparably connected with such.

Many investigators have emphasized also the great importance that colloidal chemistry has in biologic and immunologic chemistry, and there has been no lack of endeavors to explain every immunologic problem as colloidal. But some of these investigators—for instance, Traube<sup>1</sup>—have undoubtedly gone too far when they try to reduce immunology, including specificity, to pure surface processes, and such exaggerations have in many places deprived colloidal chemistry of credit.

At all events it has to be understood that the metabolism of the animal organism at the last instance is a purely chemical process, and tho the colloidal nature of the implicated substances cannot fail to produce an effect, the genuine chemical processes will take precedence. Speaking theoretically we must content ourselves with putting colloidal chemistry in the second place; but experience teaches us that where colloidal substances have been brought into contact with each other, it is their physical surface relations and electrical charges—that is, their colloidal state—which determines their quantitative relation, the chemical nature of the substances keeps in the background.

Consequently, biochemical investigations may reveal some quantitative facts belonging to the colloidal nature of the substances, but further than this we cannot as yet penetrate in spite of strong suspicions that some other process, possibly of a fermentative nature, cooperates tho hidden by the colloidal process. That is to say, studying immunologic problems we must always keep in mind the colloidal chemical relations, but we must not allow them too much weight, and whenever we have discovered what appears to be a colloidal chemical relation, we have always to ask ourselves whether this is not merely a shell that we have to crack in order to get at the real process.

\* Received for publication April 23, 1916.

<sup>1</sup> Ztschr. f. Immunitätsf., 1911, 9, p. 246.

In the following pages I have endeavored to collect some facts and theories which may help to illustrate the somewhat obscure relations between colloidal chemistry and immunology.

## I

The physical relations of the substances called colloids are best characterized as representing heterogeneous systems, that is, suspensions of solid particles in a fluid or emulsions of two fluids; but these mixtures are very intimate ones, and therefore the surfaces forming the limits between the mixed substances are extensive. But the relations of fluids at their surfaces and within their interiors are quite different. The surface of a fluid represents a tense elastic membrane, endeavoring to contract itself; this is the reason why a falling drop takes the globular form, the form in which the surface is reduced to a minimum. If I now dissolve a substance in the fluid, it will in most cases modify the surface tension, augmenting it or lessening it proportionately to the concentration. If now the dissolved substance lessens the surface tension, such being generally the case, this tension trying to get lessened draws a quantity of substance out of the surface of the fluid, thereby augmenting the surface concentration. The result may be rather a great difference between the concentration at the surface and that in the interior of the fluid.<sup>2</sup> On the surface the concentration may be so great that the substance will separate itself as a solid membrane, such as we may see at the surface of old urines or in old staining solutions.

But the surface tension does not exist only on the free surface of a fluid; it exists also on every surface forming the limit to another liquid or to a solid body. Here also we meet the augmenting concentration, and if the fluid moistens the limiting surface, the dissolved substance will fix itself on the solid body and so be eliminated from the liquid.

This is the phenomenon generally named adsorption, a process that is of great importance in all relations of colloidal chemistry. We see adsorption not only to solid bodies and to suspended and emulsified particles, but also to the smallest particles in the colloidal systems, and these particles, which themselves often can influence the surface tension, may be absorbed to solid bodies, suspensions, emulsions, or other colloid particles.

So we may have a great many different processes in the colloidal systems, and in the very complicated liquids that form blood and the

<sup>2</sup> Gibbs: *Thermodynamical Studies*. Freundlich: *Kapillarchemie*, 1909.

fluids of the body, or that result from our cultivation of bacteria in liquid media; we may expect to encounter whole series of different adsorptions, one parallel to another or antagonistic to another, forming a complicated system.

The characteristic trace by the adsorption is that the inner mechanics of the system do not change. The colloid particles keep themselves as before, their number neither augmenting nor lessening. But we may see other processes in colloidal chemistry in which such a change takes place. So we have the coagulation, or better, precipitation of a colloidal system, in which a precipitate forms itself under some certain outside influence; and we have the solution of a precipitate with the result that a coarse suspension dissolves itself under formation of a colloid solution. In both instances we see a change in the inner mechanics of the system, the number of the particles changing, several small particles agglutinating into one, or one greater particle disintegrating into several smaller. I propose to collect those two processes—in their effect great contrasts but in their nature closely related—under the name of alternations. Colloidal processes then may be considered under these groups: Adsorption, a binding in a colloidal system without change in its degree of dispersion; and Alternation, a binding that effects a thorough change in the same. (The degree of dispersion is the relation between surface and cubic measure of the particle: the more finely dispersed the substance the greater this relation.)

## II

Quantitatively we may express the law of adsorption by a formula:

$$x = k \frac{y}{(1-y)^{1/n}}$$

in which  $x$  represents the quantity of adsorbing matter;  $y$  the adsorbed part of the substance adsorbed to  $x$ , the total quantity of this equalling 1;  $1-y$  the nonadsorbed part;  $k$  a constant factor, depending first on the unities applied to  $x$  and second on the nature of the substances, the temperature, etc.; and  $1/n$  an exponential constant, always less than 1, generally between 0.8 and 0.1.

Generally the adsorption formula is expressed in another way. For example,

$$\frac{y}{x} = k' \left[ \frac{1-y}{v} \right]^{1/n}$$

in which  $v$  indicates the volume in which the reaction takes place. In this form the formula expresses that the concentration of the adsorbed substance

(y) on the adsorbing substance, (x) — namely the quotient  $y/x$  — is proportional to the concentration of the remaining substance  $1 - y/v$  potensated with  $1/\bar{n}$ . But if we always employ the identical volume in a series of experiments, the volume may be contained within a constant

$$k \left[ = \frac{k'}{v^{1/\bar{n}}} \right]$$

and may be neglected in the schematic formula. We then get

$$\frac{y}{x} = k (1 - y)^{1/\bar{n}}$$

but I have preferred to develop this formula with respect to x, thus getting the afore-mentioned formula.

It may be mentioned that most books and papers on colloidal chemistry use other letters for the quantities, as is also the case in my paper on diphtheria toxin and antitoxin. We find in the place of x, m as the adsorbing substance; in the place of y, x as the adsorbed part; in the place of 1, a as the original quantity of the substance which is adsorbed; and in the place of  $1/\bar{n}$ , q as the exponential constant. The formula then looks like this:

$$\frac{x}{m} = k (a - x)^{1/\bar{n}} \quad \text{or} \quad \frac{x}{m} = k (a - x)^q$$

Sometimes  $a - x$  is expressed as C for the remaining concentration, thus giving

$$\frac{x}{m} = k C^{1/\bar{n}}$$

and  $a - x$  may be named  $C_2$  and  $x/m$  expressed as  $C_1$  and so we ultimately get:  $C_1 = k C_2^{1/\bar{n}}$ .

But for the purely mathematical treatment of this formula it is more convenient to use the signs generally used in mathematics; that is, x, y, etc., for the variable quantities, and k, q, n, etc., for the constant ones. Also I have used y to signify the fraction of adsorbed substance, employing the original quantity as unity. In this way I have arranged to reduce quantities which are absolutely very different, to the same order, a procedure that makes the calculation and the comparison of the different figures in the different experiments much easier.

Besides the law of adsorption, another law must be mentioned, not a law of colloidal chemistry, but in form closely allied with the adsorption law and undoubtedly of importance in immunology. This is the law of partition, written thus:

$$x = k \frac{y}{1 - y}$$

This law rules the partition of a dissolved substance between two fluids that do not mix, such as oil and water. In the form which I have given to the

formula, it indicates for instance the fraction ( $y$ ) of a quantity of phenol (1) that would be extracted from the water by a certain quantity of oil ( $x$ ).

Generally this law is written thus:  $C_1 = kC_2$ , which is analogous to the last form that I stated for the adsorption formula. The importance of this formula is that in certain cases it may be absolutely identical with the adsorption formula; that is, when the molecular weight of the dissolved substance is different in the two media.

Thus, the molecular weight of benzoic acid in a solution in benzol is double its molecular weight in water; that is to say, in benzol 2 molecules join together.

If I now to a solution of benzoic acid in benzol add a certain quantity of water ( $x$ ), I shall get the formula:

$$\frac{C}{\text{water}} = k \frac{C}{\text{benzol}}$$

But if I now use the first-mentioned letters, I get:

$$\frac{C}{\text{water}} = \frac{y}{x} \quad \text{and} \quad \frac{C}{\text{benzol}} = \frac{1-y}{v} \quad \text{and thus} \quad \frac{y}{x} = k \left\{ \frac{1-y}{v} \right\}^{\frac{1}{2}}$$

or, if I develop this formula with respect to  $x$ :

$$x = kv^{\frac{1}{2}} \frac{y}{(1-y)^{\frac{1}{2}}}$$

a formula that cannot be distinguished from the adsorption formula. The only difference is that in partition the exponential constant has to be a very simple fraction, whilst in adsorption it may have almost any value less than 1.

### III

I shall now proceed to some of the endeavors made to register immunologic processes as adsorptions.

As to agglutination, Eisenberg and Volk<sup>3</sup> could establish that fixation to specific bacteria followed a formula

$$x = k \frac{y}{(1-y)^{0.667}}$$

$k$  having the value 24.7 for typhoid agglutinins and 19 for cholera vibriones. Arrhenius and Morgenroth<sup>4</sup> in a study of the fixation of hemolytic amboceptor to blood corpuscles found exactly the same relations,  $1/n$  equalling 0.667 ( $\frac{2}{3}$ ), and  $k$  equalling 18.3 for ox-blood corpuscles and 39.4 for sheep blood corpuscles.

Now it ought to be mentioned that Arrhenius<sup>5</sup> does not consider these fixations as adsorptions, but insists that they be considered as partitions. Of course it is very possible that a constant  $1/n = \frac{2}{3}$

<sup>3</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1902, 40, p. 155.

<sup>4</sup> Arb. a. d. k. Gsndhtsamte, 1904, 20, p. 559.

<sup>5</sup> Ibid. Also Immunochemie (1907) and later works.

might be interpreted in this sense. The agglutinin ought then to change its molecular weight from 1 to 1.5; that is 3 molecules of agglutinin unite, when they enter the bacterium, into 2. And the circumstance that in two different immune processes we find the same value, will of course not fail to give valor to this conception. But in spite of this, it appears to me somewhat artificial to look for an explanation outside of adsorption, which undoubtedly is the most natural explanation. But Arrhenius urges against the possibility of an adsorption some experiments by Madsen with the fixation of coli-agglutinin in which the constant  $1/n$  varied considerably and once was greater than 1, being 1.25. This fact is hardly compatible with an adsorption, where  $1/n$  has to be less than 1; but these experiments cannot any more be reconciled to the theory of partition; then the molecular weight ought to be as constant as the exponential constant in the adsorption formula, and we cannot admit a change in the molecular weight sometimes to the smaller ( $1/n > 1$ ), and at other times to the greater, as when  $1/n$  is less than 1. We have to presume that the experiments of Madsen were executed under special circumstances which influenced their course. As considered by Arrhenius they do not permit a final conclusion.

Lastly I should mention my own endeavors to explain the fixation of toxin to antitoxin as an adsorption.<sup>6</sup>

Zangger<sup>7</sup> and Bilz, Much and Sieber<sup>8</sup> have suggested physical relations as predominant in the fixation and neutralization of toxin by antitoxin. Whilst Zangger limits himself to general considerations, Bilz and his collaborators enter more into details. They studied the fixation of the toxin to suspended anorganic substances. As they found that such fixation, which is undoubtedly an adsorption, took place, and believed that they had found that the toxin was made harmless by this process, they constructed on these experiments a purely physical theory of the neutralization of toxin.

In my experiments I could not confirm these observations. I found the toxin adsorbed to an anorganic suspended substance quite as harmful to guinea-pigs as before. The only difference observed was that its resorbability was hindered and its effect therefore more locally limited.

But the adsorption of toxin and of antitoxin to hydroxid of iron I was able to follow in all its phases, and I found that it followed

<sup>6</sup> Ztschr. f. Hygiene, B. 71.

<sup>7</sup> Centralbl. f. Bakteriolog., I, O., 1903, 34, p. 428.

<sup>8</sup> Behring's Beiträge, 1903, No. 10, p. 30.

strictly the adsorption formula. The adsorption of toxin gave  $1/n$  equal to 0.278 and  $k$  equal to 15.4, whilst the fixation of toxin to anti-toxin gave to the same constants the values of 0.312 and 118.2. Later I calculated a number of other experiments by the adsorption formula and generally found good agreement especially in the last part of the curve where the toxin is saturated from 50 to 100%. In the first part of the curve the agreement is not always good, sometimes rather bad; but the method for determining the neutralization of the toxin in this part of the curve (up to 50%) is so inaccurate that we can hardly expect an exact conformity with any formula.

Arrhenius also denies the adsorption nature of this process, for which he urges a purely chemical nature; namely, the application of the law of Guldberg and Waage. Against this conception we may plead the firmness of the fixation and its lack of reversibility, its successive fastening, and the phenomenon of Danysz. All these features are essentially characteristic of adsorptions and added to the recognized colloidal nature of toxin and antitoxin ought to make it exceedingly probable that the process is an adsorption.

But the adsorption as such does not neutralize the toxin. Here we have to look for something behind the colloidal chemistry. Two possibilities suggest themselves to me: a purely chemical binding within the colloid particle or a fermentative decomposition of the toxin brought about by the antitoxin in some specific way. Experiments to this effect have not as yet been made, and this part of the question remains open.

#### IV

As to adsorption in immunology it is thus possible to enumerate several facts indicating the importance of the process. But if we now take up the other colloidal process, the alternation, we find ourselves on a quite new ground. First, as mentioned, alternation is a new conception. Second, we have no researches whatever that try to register such processes as hemolysis in colloidal chemistry.

The most important example of alternation in immunology is hemolysis. The S-formed course of this process is well known and very characteristic. Many have endeavored to explain its nature by this course but the result has been far from satisfactory.

A curve such as this is however not without analogies. Simon<sup>9</sup> has communicated some researches concerning the precipitation of albuminous substances with metal salts, in which we meet with exactly the same curve.

<sup>9</sup> Arch. di fisiologia, 1910, 8, p. 373.

The theoretic formula of a curve such as that mentioned is

$$y = \frac{x^n}{x^n + k}$$

(This curve with  $n$  equalling 2 is known in mathematics as the witch of Agnesi; applied here  $n$  always is greater than 2 up to 10 and more.) In this formula  $y$  ought to be the degree of hemolysis,  $x$  the quantity of hemolysin, and  $k$  and  $n$  constants.

If I develop this formula to  $x$  I get

$$x = k \left\{ \frac{y}{1-y} \right\}^{1/n}$$

A formula that as to its formals is closely related to the adsorption formula

$$x = k \frac{y}{(1-y)^{1/n}}$$

The only difference is that in the alternation formula the exponential constant is applied to the whole fraction  $y/1-y$  instead of to the denominator only as in the adsorption formula.

This formal analogy to a well known colloidal chemical formula gives, a priori, a certain probability, first to the formula itself, and second to the conception of the processes whereby it may be applied as purely colloidal. I have calculated a great many experiments on hemolysis with this formula and it has always appeared that if the experiment allows the tracing of any regular curve at all, the alternation curve coincides excellently with it. The more exact the experiment, the closer the fitting.

The calculation of an experiment by the alternation formula is accomplished, as regards its principles, by the same method as was indicated by Ostwald for calculations with the adsorption formula. The method consists in replacing the direct values by their logarithms.

The adsorption formula

$$x = k \frac{y}{(1-y)^{1/n}}$$

written in the common way

$$\frac{y}{x} = \frac{1}{k} (1-y)^{1/n}$$

and logarithmated gives

$$\log \frac{y}{x} = \log \frac{1}{k} + \frac{1}{n} \log (1-y).$$

But a formula like this is identical with the genuine formula of first degree:

$$u = a + bv$$

If I graph this formula, putting  $v$  as abscisses and  $u$  as ordinates, I get at a straight line; the tangent to the inclination angle of this line is equal to  $b$ . It will be seen that in logarithmic formula  $\log y/x$  corresponds to  $u$ ,  $b$  to  $1/n$ , and  $\log (1-y)$  to  $v$ . By drawing up  $\log y/x$  and  $\log (1-y)$  as ordinates and abscisses respectively I have the opportunity of finding  $1/n$  by determining the tangent to the inclination angle.

The constant  $k$  has to be calculated in the ordinary way of equations.

Applying these principles to the alternation formula, I get

$$x = k \left[ \frac{y}{1-y} \right]^{1/n}$$

or logarithmated:

$$\log x = \log k = \frac{1}{n} \log \left[ \frac{y}{1-y} \right]$$

Here also I shall have to draw up the curve with  $\log x$  and  $\log y/1-y$  as ordinates and abscisses respectively,  $1/n$  being as before the tangent to the inclination. Here the calculation of  $k$  is much easier inasmuch as when  $y$  equals 0.5,  $y/1-y$  always equals 1,  $k$  thus being equal to  $x$ .

## V

I now shall bring to notice some experiments on hemolysis calculated by the alternation formula.

First, I should mention some experiments of Madsen and Teruuchi communicated by Arrhenius.<sup>10</sup> These experiments, which were made with great accuracy, represent the average of many single experiments. The course of the curve is therefore very regular. In Table 1 are the calculations by the alternation formula, in Chart 1 the logarithmic curve representing a straight line, and in Chart 2 the ordinary curve. The calculated curve is traced and the values found by Madsen and Teruuchi are indicated by crosses.

It will be seen that in the logarithmic curves there is for the first part of the curves a rather weak conformity; but in the genuine curve on Chart 2 no trace of disagreement can be found. The reason is that for the first and last parts of the curve a very slight change in  $y$  gives a very great effect in  $y/1-y$ ; thus the disagreement in the logarithmic curve is only an apparent one, which does not exist in reality.

But the last two points have given too small values in the calculations; it might be said that when hemolysis is very near to 1, its determination is rather difficult; but it may be possible that the formula, which only asymptotically nears the value of  $y$  to 1, whilst the

<sup>10</sup> Meddelanden fran k. vetenskapsakademins Nobelinstitut, Vol. 2, No. 39.

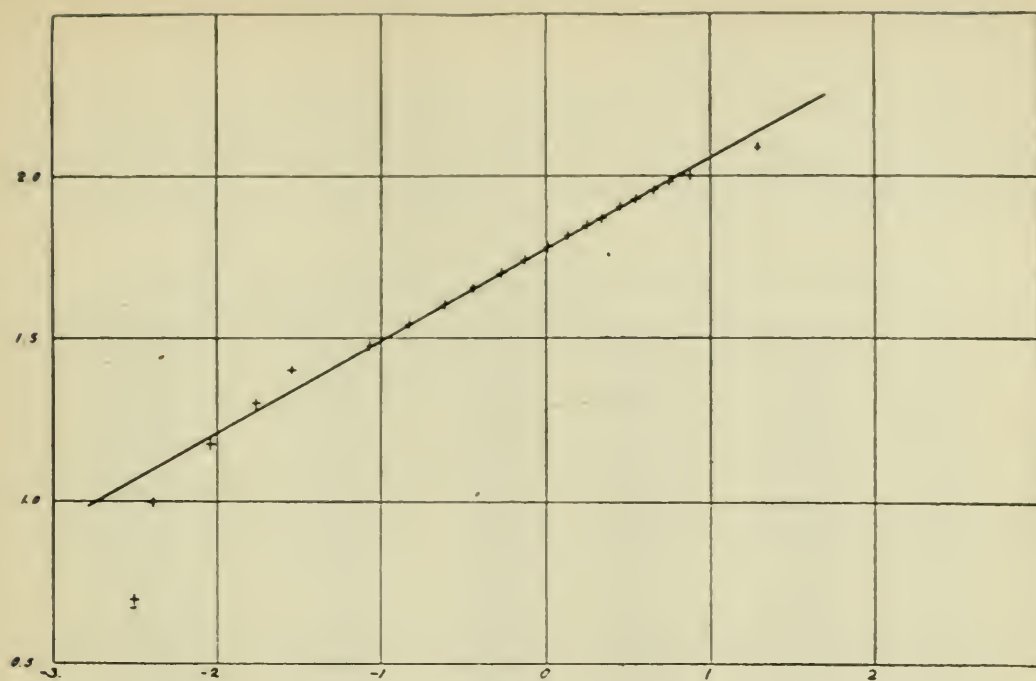


Chart 1

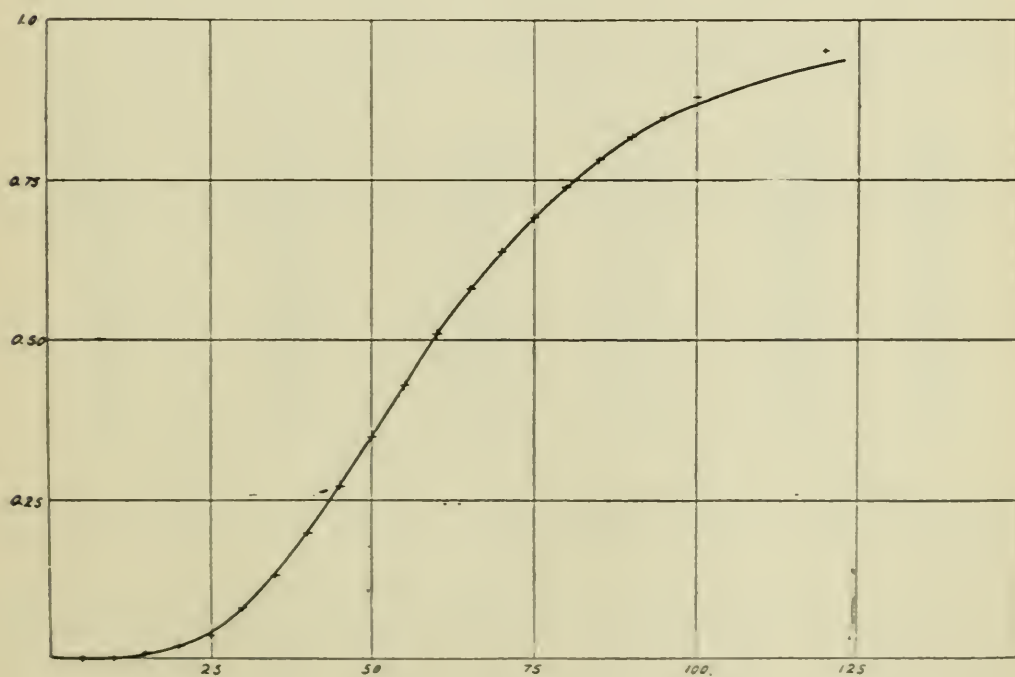


Chart 2

Charts 1 and 2. The logarithmic and ordinary curves for Madsen and Teruuchi's experiments on hemolysis calculated by the alternation formula. The values found by Madsen and Teruuchi are indicated by crosses.

hemolysis in reality gives a fairly well-determined value of  $x$  for  $y=1$ , here needs correction.

Table 2 illustrates an experiment with amboceptor and complement executed by Leschly,<sup>11</sup> and in Charts 3 and 4 are represented the logarithmic and ordinary curves. Table 3 gives an experiment of

TABLE 1  
HEMOLYSIS WITH VIBRIOLYSIN, ACCORDING TO MADSEN AND TERUUCHI

x	y Found	y Calculated	Log x	Log y/1-y Found	Log y/1-y Calculated
5	0.003	0.001	0.699	0.497—3	0.000—3
10	0.004	0.002	1.000	0.603—3	0.260—3
15	0.009	0.007	1.176	0.953—3	0.890—3
20	0.017	0.020	1.301	0.238—2	0.330—2
25	0.034	0.037	1.398	0.457—2	0.690—2
30	0.080	0.085	1.477	0.939—2	0.960—2
35	0.130	0.137	1.544	0.176—1	0.200—1
40	0.200	0.200	1.602	0.398—1	0.398—1
45	0.270	0.270	1.635	0.568—1	0.568—1
50	0.350	0.350	1.699	0.732—1	0.732—1
55	0.430	0.430	1.740	0.877—1	0.877—1
60	0.510	0.510	1.778	0.017	0.017
65	0.580	0.580	1.813	0.141	0.141
70	0.640	0.640	1.845	0.250	0.250
75	0.690	0.690	1.875	0.348	0.348
80	0.740	0.740	1.903	0.455	0.455
85	0.780	0.780	1.929	0.550	0.550
90	0.820	0.820	1.954	0.659	0.659
95	0.850	0.850	1.978	0.753	0.753
100	0.880	0.867	2.000	0.875	0.810
120	0.950	0.930	2.079	1.297	1.120

Vol. = 10 c.c.  $x$  = amount of lysin in cmm.  $y$  = degree of hemolysis.  $1/n = 0.286$

TABLE 2  
HEMOLYSIS WITH COMPLEMENT ACCORDING TO LESCHLY, AVERAGE OF 4 DETERMINATIONS

x	y Found	y Calculated	Log x	Log y/1-y Found	Log y/1-y Calculated
0.006	0.110	0.054	0.788—3	0.093—1	0.75—2
0.007	0.160	0.107	0.845—3	0.281—1	0.08—1
0.008	0.200	0.190	0.903—3	0.398—1	0.37—1
0.009	0.280	0.282	0.953—3	0.590—1	0.61—1
0.010	0.400	0.407	0.000—2	0.824—1	0.83—1
0.011	0.500	0.503	0.042—2	0.000	0.06
0.012	0.610	0.625	0.079—2	0.194	0.22
0.013	0.680	0.716	0.114—2	0.328	0.40
0.014	0.800	0.807	0.147—2	0.602	0.57
0.016	0.890	0.890	0.204—2	0.909	0.91
0.018	0.930	0.930	0.255—2	1.128	1.13
0.020	0.950	0.954	0.301—2	1.279	1.32
0.022	0.970	0.972	0.343—2	1.510	1.54
0.024	0.980	0.980	0.380—2	1.695	1.70

$x$  = amount of complement in c.cm.  $y$  = degree of hemolysis.  $1/n = 0.191$ .

Teruuchi<sup>12</sup> with NaOH, the curves of which are traced in Charts 5 and 6.

There is a point in these curves that seems to confirm very strongly the correctness of the formula. It will be seen that in all of them the first amount of hemolysin does not have any apparent effect at all. Only after the addition of a certain quantity can we observe any hemo-

<sup>11</sup> Studier over Komplement, Aarhus, 1914, p. 22 (average of four determinations).

<sup>12</sup> Ztschr. f. Immunitätsf., 1909, 1, p. 351.

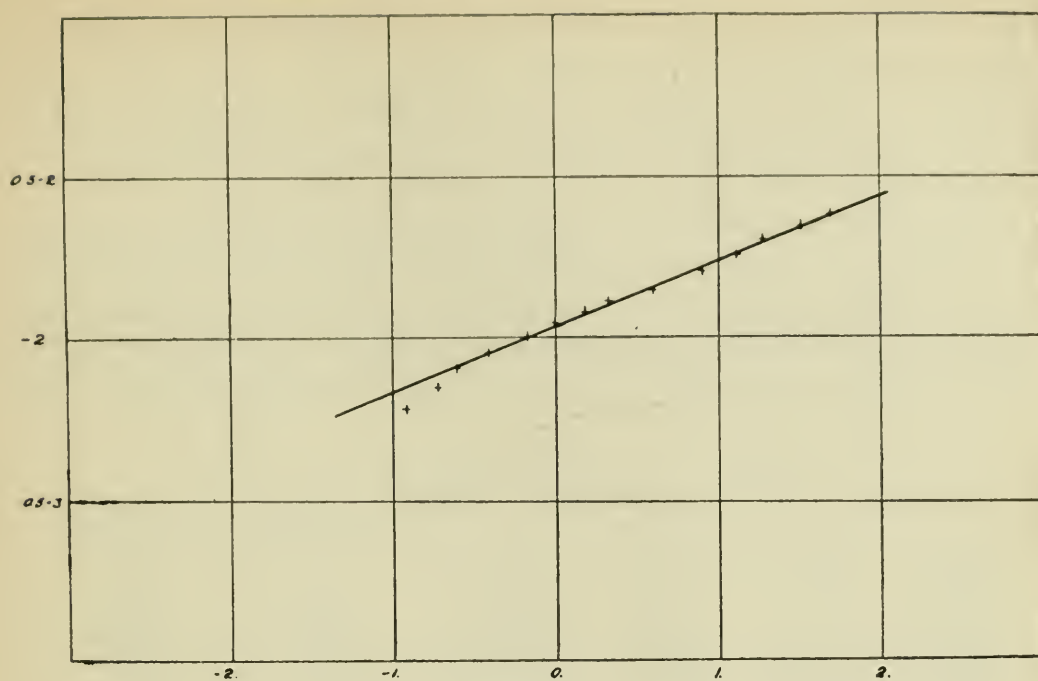


Chart 3

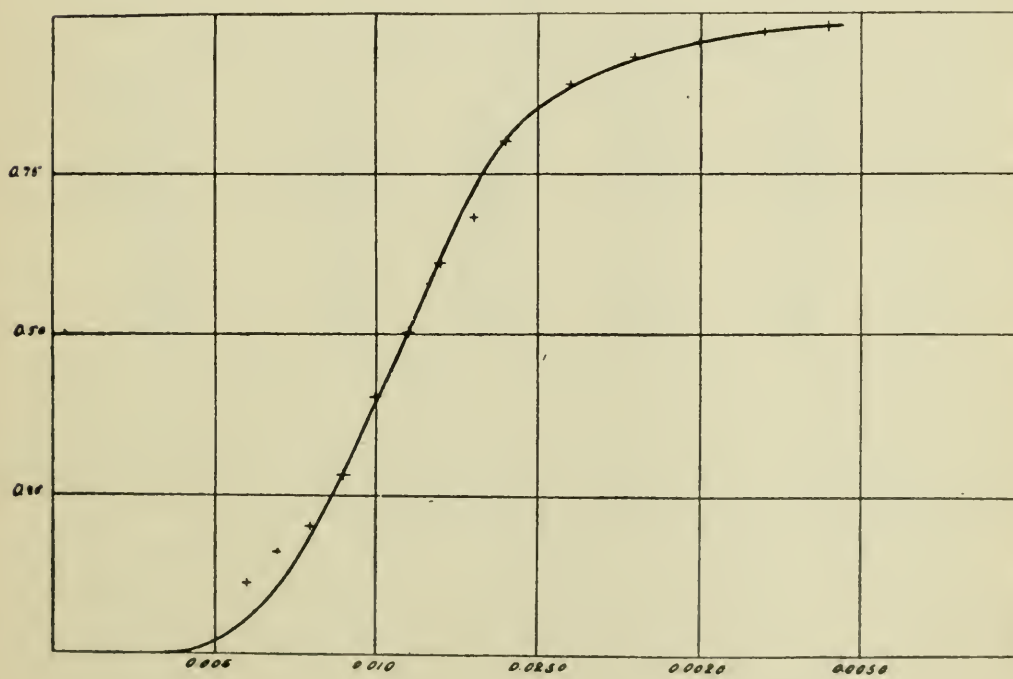


Chart 4

Charts 3 and 4. The logarithmic and ordinary curves for Leschly's experiments with amboceptor and complement.

lysis. This fact has been rather hard to explain, the investigators always believing that some foreign substance fixes some part of the hemolysin. With the adoption of the alternation formula this question disappears completely, the said course of the curve being explained by the particulars of the law.

But if we now proceed to the study of the velocity of the reaction of hemolysis, we encounter a fact that at first glance appears very strange. The velocity of reaction follows exactly the same law as the reaction with ascendent quantities of hemolysin. In Table 4 and in Charts 7 and 8 I have given the results of an experiment with  $n/100$

TABLE 3  
HEMOLYSIS WITH NaOH ACCORDING TO TERUUCHI

x	y Found	y Calculated	Log x	Log y/1-y Found	Log y/1-y Calculated
0.065	0.00	—	—	—	—
0.080	0.02	0.02	0.903—2	0.301—2	0.180—2
0.100	0.12	0.12	0.000—1	0.113—1	0.113—1
0.110	0.24	0.24	0.042—1	0.500—1	0.500—1
0.120	0.43	0.42	0.079—1	0.879—1	0.860—1
0.130	0.60	0.61	0.114—1	0.176	0.200
0.140	0.72	0.77	0.146—1	0.410	0.520
0.150	0.90	0.86	0.176—1	0.955	0.800
0.160	0.92	0.93	0.204—1	1.111	1.111
0.180	0.97	0.97	0.255—1	1.510	1.580
0.200	1.00	—	—	—	—

x = amount of NaOH. y = degree of hemolysis.  $1/n = 0.1046$ .

TABLE 4  
VELOCITY OF REACTION OF HEMOLYSIS WITH NaOH N/100

x	y Found	y Calculated	Log x	Log y/1-y Found	Log y/1-y Calculated
12	0.05	0.102	1.079	0.724—2	0.055—1
14	0.14	0.149	1.146	0.202—1	0.240—1
16	0.20	0.200	1.204	0.398—1	0.398—1
18	0.26	0.251	1.255	0.545—1	0.525—1
21	0.35	0.338	1.322	0.732—1	0.710—1
24	0.42	0.415	1.380	0.859—1	0.850—1
28	0.51	0.517	1.447	0.017	0.030
32	0.62	0.605	1.505	0.203	0.185
36	0.69	0.673	1.556	0.348	0.315
42	0.75	0.758	1.623	0.477	0.495
48	0.81	0.820	1.681	0.630	0.660
60	0.90	0.890	1.778	0.955	0.905

Temperature = 18°. x = time in minutes. y = degree of hemolysis.  $1/n = 0.375$ .

NaOH, in which the conformity between found and calculated values is very remarkable. The only explanation of this is that equal amounts of hemolysin come into action in every unity of time.

This might perhaps be explained by some experiments made by Arrhenius, who found that the fixation of hemolysin to blood corpuscles follows the simple partition law, and he gives a table which shows a very good conformity to this law. This is very interesting because it is difficult to explain this fixation as an adsorption, which is a process that always takes place with great velocity, whilst the velocity

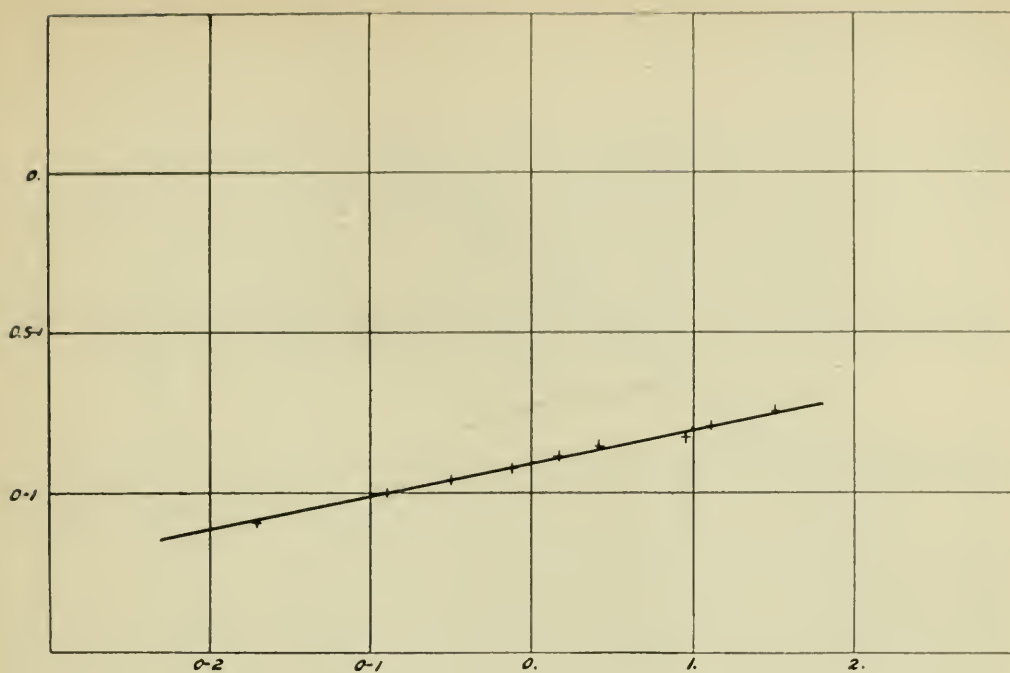


Chart 5

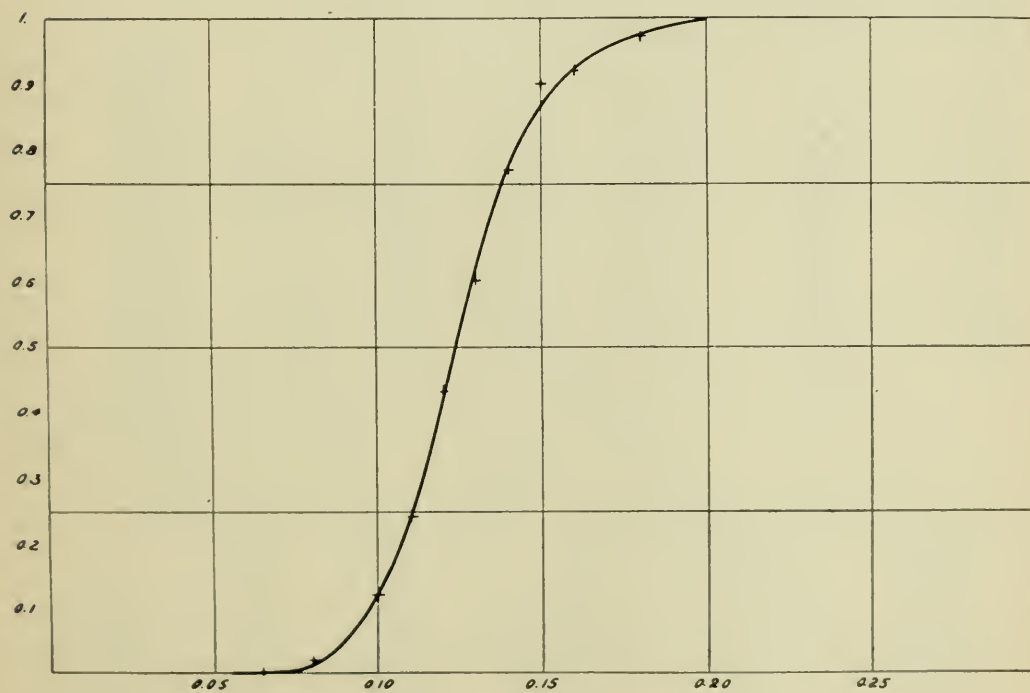


Chart 6

Charts 5 and 6. The logarithmic and ordinary curves for Teruuchi's experiments with NaOH.

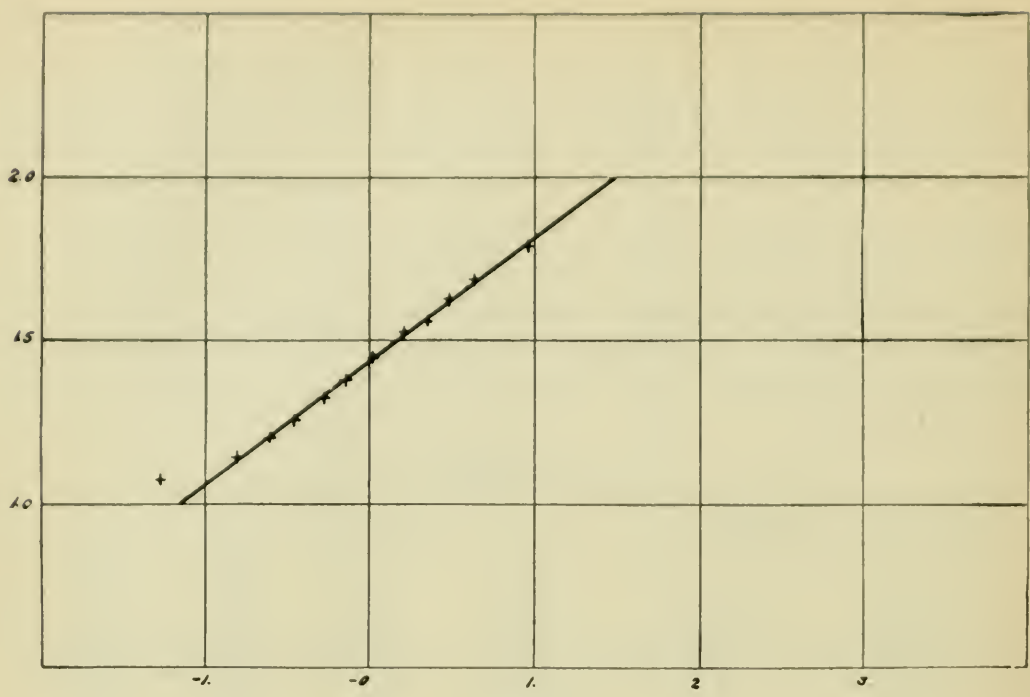


Chart 7

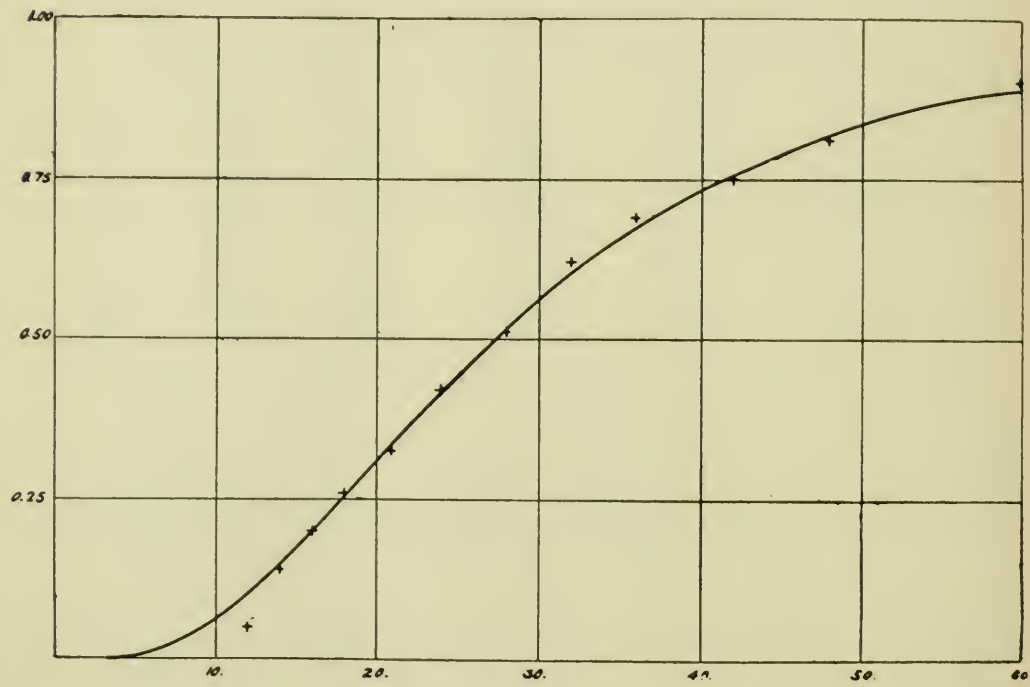


Chart 8

Charts 7 and 8. The logarithmic and ordinary curves for an experiment with  $n/100$  NaOH.

of the binding of hemolysin, whether it is vibriolysin, complement, or NaOH, always takes place with a rather slow, at least very easily measured velocity.

As a result of these investigations of hemolysis it may be said that its quantitative parts are thoroughly explained by the alternation law. The nature of hemolysin is so far irrelevant and only gives some difference in the constants. That the alternation law is purely a colloidal chemical law, will now be shown by demonstrating its relation to the coagulation of albuminous substances.

## VI

I shall communicate an experiment on coagulation (precipitation) of egg albumin with silver nitrate.

This reaction is well adapted to quantitative experiments, as the precipitation takes place very promptly, is at once complete, and, the precipitate formed being a very solid and heavy one, its further treatment on that account is easy. But in calculation we at once encounter the difficulty that the reaction is not a real one, but is made up of

TABLE 5  
COAGULATION OF EGG ALBUMIN WITH  $\text{AgNO}_3$

$x'$	$y'$	$x$	$y$ Found	$y$ Calculated	$\text{Log } x$	$\text{Log } y/1-y$ Found	$\text{Log } y/1-y$ Calculated
1.0	0.0204	—	—	—	—	—	—
2.0	0.0392	—	—	—	—	—	—
2.5	0.0498	—	—	—	—	—	—
3.0	0.0661	0.4	0.008	0.001	0.602—2	0.906—3	0.200—3
3.5	0.1032	0.9	0.100	0.050	0.954—1	0.045—1	0.750—2
4.0	0.1968	1.4	0.285	0.280	0.146	0.600—1	0.590—1
4.2	0.2777	1.6	0.440	0.440	0.204	0.893—1	0.893—1
4.4	0.3326	1.8	0.550	0.550	0.255	0.087	0.087
4.6	0.3874	2.0	0.660	0.660	0.301	0.288	0.288
4.8	0.4352	2.2	0.750	0.750	0.342	0.477	0.477
5.0	0.4712	2.4	0.820	0.816	0.380	0.695	0.640
5.6	0.5120	3.0	0.900	0.917	0.477	0.955	1.040
6.0	0.5360	3.4	0.950	0.954	0.531	1.279	1.320
6.5	0.5458	3.9	0.970	0.974	0.591	1.510	1.560
7.0	0.5558	4.4	0.990	0.986	0.643	1.996	1.830
8.0	0.5610	5.4	1.000	—	—	—	—

$x' = \text{c.c. AgNO}_3 \text{ in } 1.5\% \text{ solution, } x = x' - 2.6. \quad y' = \text{weight of precipitate.}$   
 $y' - 0.0518$

$y = \frac{y' - 0.0518}{0.561 - 0.0518} \quad 1/n = 0.226.$

TABLE 6  
COAGULATION OF SERUM ALBUMIN WITH  $\text{CuSO}_4$  IN SOLUTION 2/5 N

$x$	$y$ Found	$y$ Calculated	$\text{Log } x$	$\text{Log } y/1-y$ Found	$\text{Log } y/1-y$ Calculated
0.0200	0.05	0.027	0.301—2	0.724—2	0.46—2
0.0233	0.07	0.078	0.367—2	0.824—2	0.93—2
0.0253	0.10	0.137	0.402—2	0.045—1	0.20—1
0.0266	0.18	0.162	0.425—2	0.342—1	0.32—1
0.0286	0.23	0.262	0.457—2	0.476—1	0.55—1
0.0300	0.42	0.334	0.477—2	0.859—1	0.70—1
0.0333	0.66	0.498	0.522—2	0.288	0.98—1
0.0366	0.70	0.643	0.564—2	0.368	0.26
0.0400	0.73	0.759	0.602—2	0.432	0.51
0.0533	0.95	0.958	0.729—2	1.279	1.36
0.0666	0.99	0.990	0.823—2	1.996	1.99

$x = \text{c.c. of solution, } y = \text{precipitate in fraction of the total precipitation. } 1/n = 0.148.$

purely chemical and colloidal chemical factors. We therefore find that the first part of the reaction is absolutely rectilinear, corresponding to a precipitation of silver chlorid and other chemical compounds of silver, and first after having saturated the substances that enter into chemical connections with the silver we find a real alternation. In the curve we must on this account subtract from the ordinate and from the abscissae the part that corresponds to the rectilinear part of the curve, and calculate only the rest of it. In Chart 9 I have shown the total curve that up to a value of  $x$  of 2.6, is perfectly straight. To this value of  $x$  corresponds an absolute  $y = 0.0518$ , and these values have

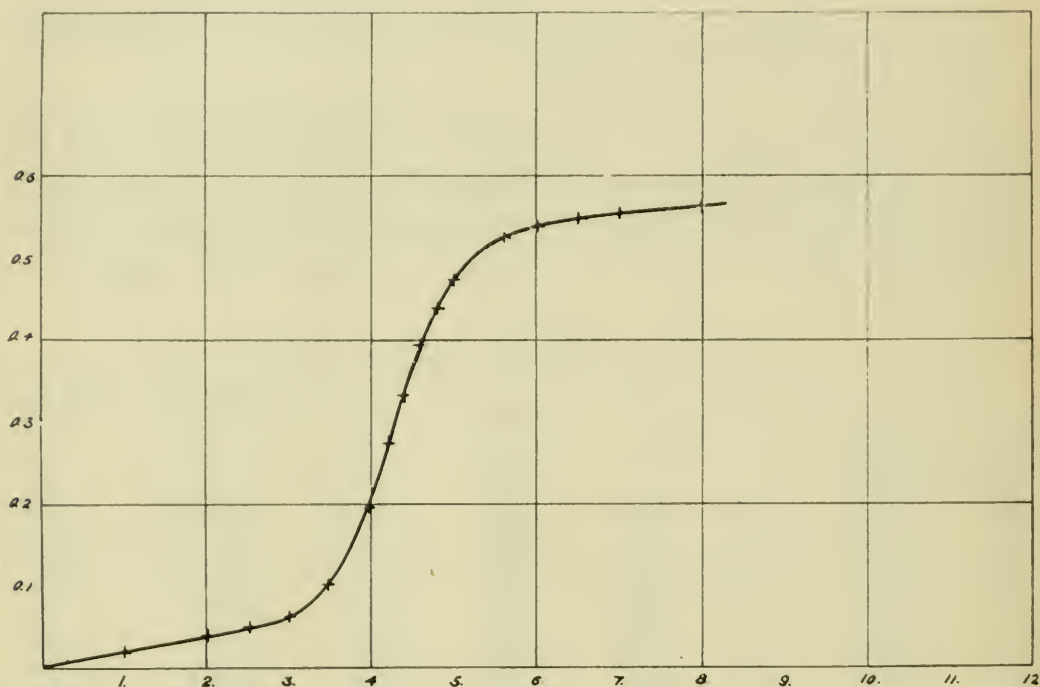


Chart 9. Curve for an experiment on coagulation (precipitation) of egg albumin with silver nitrate.

to be subtracted from  $x$  and  $y$ . The value of  $y$  got in this way has been divided with the value of the complete precipitation also reduced by subtraction so as to get the relative fraction of  $y$ .

The technic was as follows:

In a number of centrifuge tubes, the weight of which had been precisely determined, were placed increasing amounts of a solution of silver nitrate 1.5%. By experiment I found that 8 c.c. of this solution were sufficient for the complete precipitation of 10 c.c. of the solution of egg albumin. The volume of the silver solution was brought up to 10 c.c. in every tube and the 10 c.c. of the albumin solution added. After a few minutes the precipitate was well formed. The tubes were then centrifugated, the precipitate twice washed

with distilled water, which was decanted, and the tube dried for 24 hours at 120°. Ultimately the tube was weighed for the second time, and so the exact weight of the precipitate was determined.

It will be seen that the conformity between the calculated curve and the reduced experimental curve is a very close one, with the exception of the first point, where a value too great has been found for  $y$ . Probably here the last part of chemical precipitate mixes itself with the beginning of the colloid precipitation.

The precipitation of albumin solutions with copper salts has the advantage of being purely colloidal, but is not so regular as precipitation with silver salts. The reaction continues for a long time so that if we examine the precipitate at once we get entirely different values from those we should get after some hours. I refer to the experiment of Simon<sup>9</sup> with  $\text{CuSO}_4$  and serum albumin. The conformity is not a very good one, but sufficient to prove the validity of the law (see Table 6 and Charts 12 and 13).

Agglutination is probably a purely colloidal phenomenon, and it would be very interesting if we should apply the alternation formula to it. But the technical difficulties of the determination are rather great, and as yet I have not attained any curves.

## VII

There is a phase of agglutination as of hemolysis (particularly with  $\text{NaOH}$ ) that ought to be considered. This is that the process is not concluded at a certain time, but runs along tho with decreasing velocity, as presumed by the formula of the velocity of reaction. The hemolysis with complement or with vibriolysin comes to a stop at half an hour at 37 C., but the hemolysis with  $\text{NaOH}$  shows a tendency to proceed for a long time. The result is that if I examine the hemolytic curve with increasing quantities of  $\text{NaOH}$  at different times, the curve shows a tendency to rise as shown in Chart 14. The curve begins with the form  $b$  and terminates by taking the form of  $a$ . In the case of agglutinations we find the same to be true.

Considered from a purely mathematical point of view, this signifies a change in the constants of the formula, both becoming smaller as the curve is rising.

This brings us up to the question of the sense of the constants. As related in the foregoing the constant  $k$  in the adsorption formula depends, first on the unity of  $x$ , second on the constant  $1/n$ ; but these being eliminated, it gives us a direct measure for the adsorption power

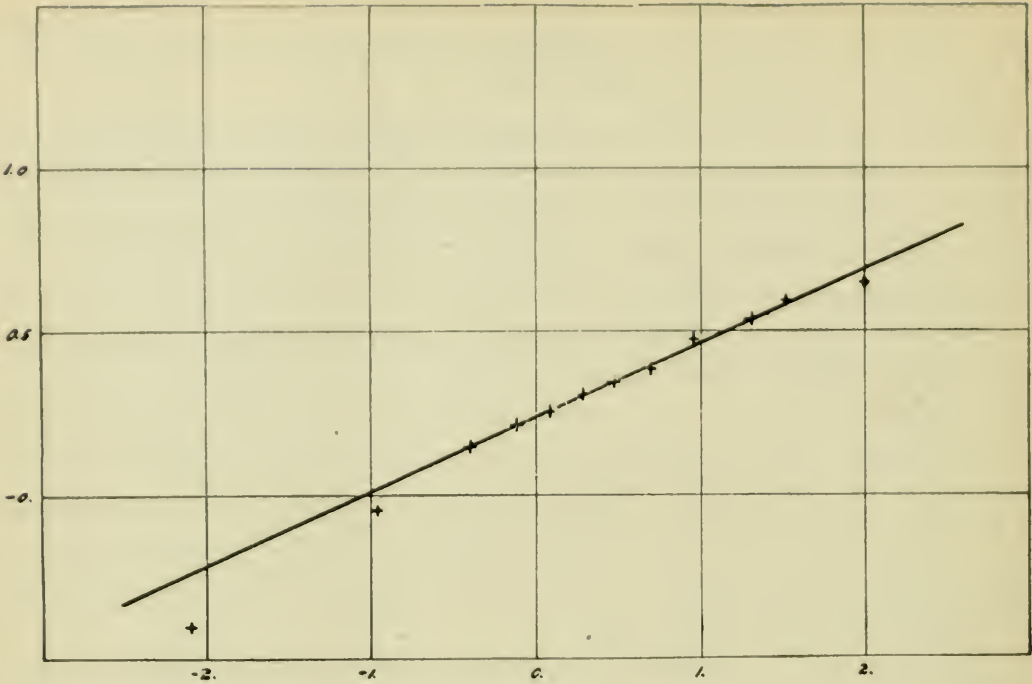


Chart 10

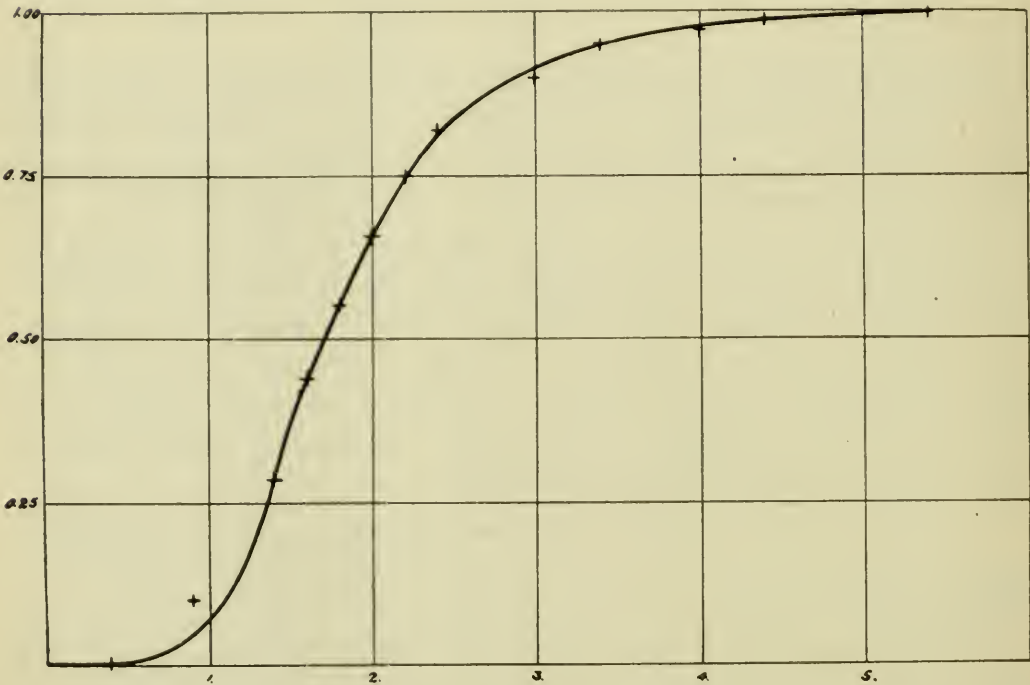


Chart 11

Charts 10 and 11. The calculated curve and the reduced experimental curve for an experiment on coagulation (precipitation) of egg albumin with silver nitrate.

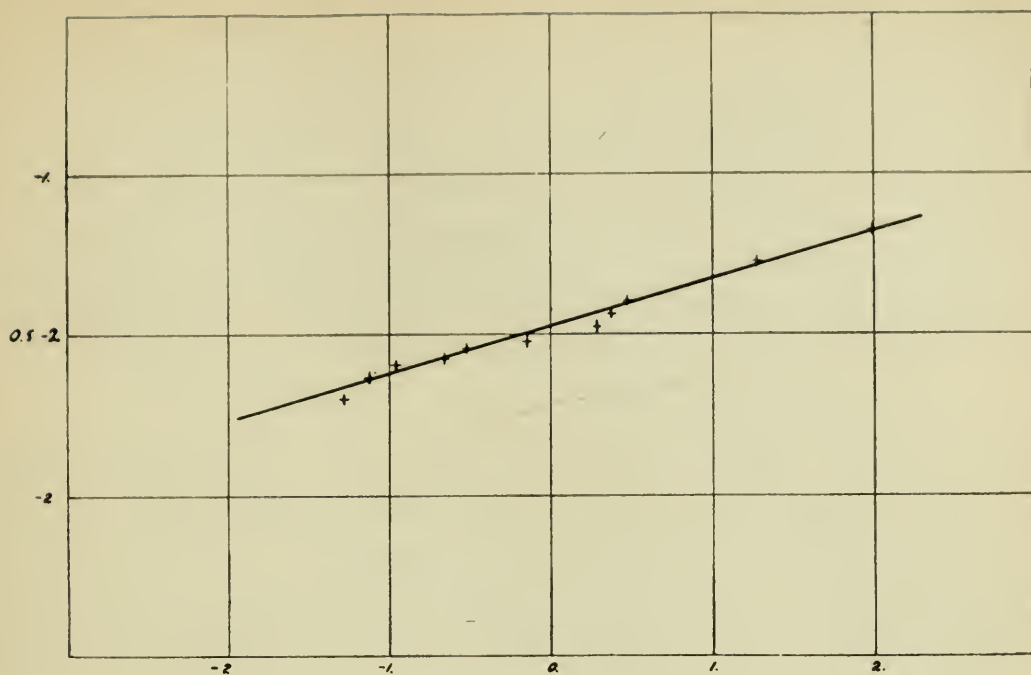


Chart 12

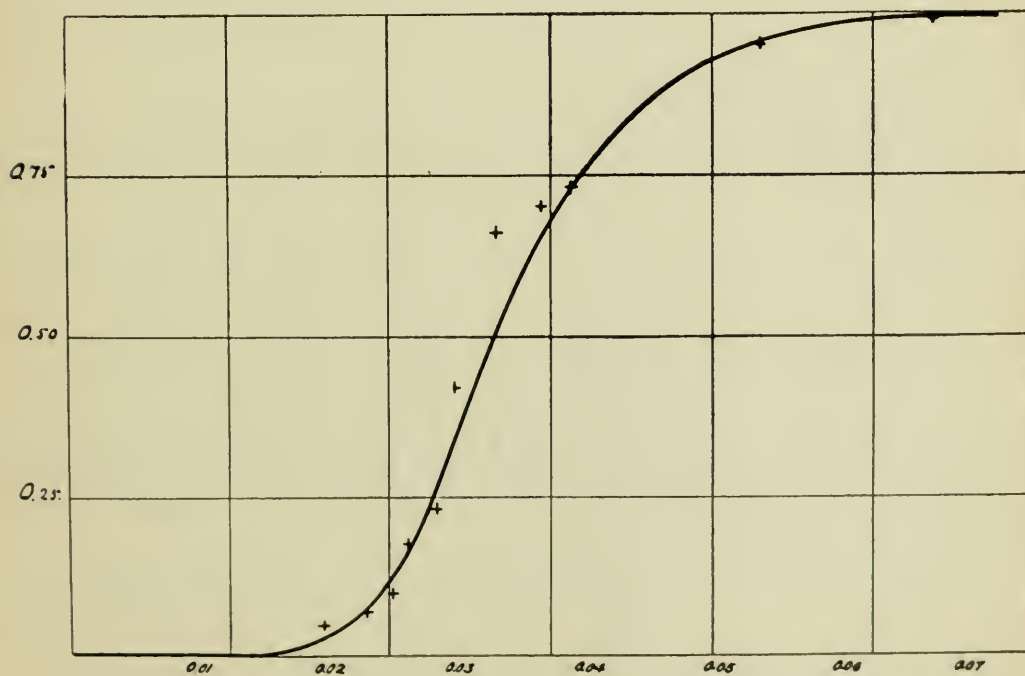


Chart 13

Charts 12 and 13. Curves for Simon's experiments with  $\text{CuSO}_4$  and serum albumin.

of  $x$ . In the alternation formula it is entirely independent of  $1/n$  and directly indicates the amount of  $x$  that gives  $y = 0.50$ .

But not so with  $1/n$ . This constant that in the adsorption formula is of importance for the determination of the adsorptive power is in the alternation formula only an indicator of the difference between the amount of the hemolysin (or coagulating substance) that gives an alternation of 100% (approximately) and the amount that is apparently without effect. A greater  $1/n$  (nearer to 1) gives a large difference and a more level course to the curve. A smaller  $1/n$  (nearer to 0)

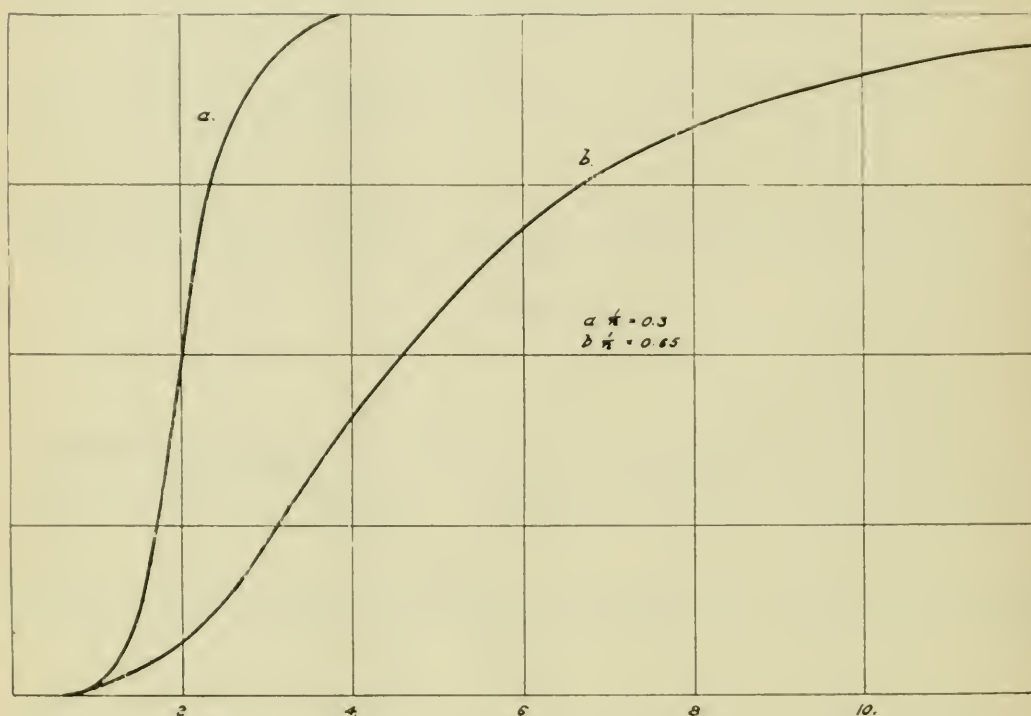


Chart 14. Curve of the progress of hemolysis with NaOH. The curve begins with the form b and terminates by taking the form a.

gives a steeper curve and a small difference between  $x$  for  $y=1$  and the maximal value of  $x$  for  $y=0$ . If with a constant  $k$  I trace a lot of theoretical curves with different  $1/n$ , all these curves intercross in a point where  $y = 0.5$  and  $x = k$ .

But as yet it has not been possible to find a plausible explanation of the constant  $1/n$ . It might be an obvious conclusion that it has a relation to the degree of dispersion of the colloidal systems, but by trying to develop this theory I found myself entangled in such a net of mutually contradictory facts that it had to be abandoned. Neither has

the endeavor to relate the colloidal chemical laws to the law of Guldberg-Waage given any encouraging results.

But I suppose we shall not err when we interpret the whole of the immunologic processes in the matter of their quantitative relations as colloidal processes, following partially the adsorption law, partially the alternation law, with the exception of some few that follow the partition law. Theoretically we may think of other of the possibilities which may present themselves as analogous formulas,  $x$  for example:

$$x = \frac{y^{1/n}}{1 - y} \quad \text{and} \quad x = \frac{y^{1/n}}{(1 - y)^{1/q}}$$

but these possibilities have as yet not been examined. The first gives a curve with a turning point much nearer to the abscissae than the alternation curve, to which it has some resemblance; but I have as yet seen no process that is likely to follow it. The other is a curve of great flexibility, and it may be that it is in reality the curve of adsorption as well as of alternation, giving by slight differences in one of the exponential constants from the adsorption or alternation formula the corrective element which these formulas sometimes need to make them fit closely to the experiments. But the calculation of an experiment to this formula needs special resources, and so I have not been able to verify these possibilities.

### VIII

But now we encounter a last question: Have we in reality solved the immunologic problem by referring fixation to colloidal formulas? And do these formulas satisfy the qualitative side of the question? I regret to say that I do not think so. First of all we get no explanation at all of specificity through the colloidal chemical theory. The adsorptions as well as the alternations are not specific processes. The adsorption of diphtheria toxin may happen to every positive colloid or suspension: but neutralization takes place by fixation to antitoxin only. We may have a nonspecific hemolysis for instance in NaOH and with vibriolysin, but that does not explain anything about the specific immune body, the amboceptor, and the necessity of its previous fixation to the blood corpuscle for hemolysis by complement, as little as the adsorption of the amboceptor to the blood corpuscle explains anything.

No endeavor at an explanation of specificity on the basis of colloidal chemistry has been successful, and the only thing that we know about specificity, namely, that it seems to be in some way connected with

aromatic aminoacids in the antigen molecule, is of a purely chemical, not colloidal chemical, nature.

I think that a portion of the truth may be found in a series of facts brought to light in the last few years. This is the investigation of blood ferments.

Inaugurated in 1912 by Abderhalden<sup>13</sup> this investigation has been, as far as I have been able to find in literature, mostly the work of North-American investigators.<sup>14</sup>

Abderhalden ascertained that after injection of some substances there appeared in the blood ferments which could decompose the injected substance. This was the result not only with genuine antigens (albumins) but also with fats and carbohydrates.

The case has been carefully studied especially as to the effect of the albuminous substances. It appears that not only albumin foreign to the species may have the effect of provoking the fermentative effects in the serum, but that the entrance of albumin of the proper organism but foreign to the blood may have the same effect. For instance, we see ferment in the blood for the albumin of the placenta (known as the Abderhalden reaction) due to the transit of placental substances into the blood during pregnancy. Abderhalden himself was of the opinion that these ferments were formed under the influence of the antigen, or at least were set free from the organ cells and passed into the blood under this influence, more or less in analogy with genuine antibodies, from which, however, they were in many respects different.

But the North-American workers have made new and important researches on this point, which, tho not yet concluded, have in an eminent degree widened our views as to generalities in immunology.

The experiments of Jobling, Petersen and Eggstein, as well as of Bronfenbrenner, show that the placental tissue was not decomposed by the ferment, but on the contrary is augmented in weight as well as in nitrogenous substance, and if the placental tissue is digested in the cold with the serum and then separated from it by the centrifuge and both parts, placenta and salt solution, kept at 37 C., the serum is split and the placenta not. The only explanation of this is that the ferment exists in the serum but cannot develop its effect because of the presence of some body with antifermentative effect. This antiferment being adsorbed by the placenta, the ferment is free and able to digest the serum albumin substance.

<sup>13</sup> Schutzfermente des tierischen Organismus, 1912.

<sup>14</sup> Jobling, Petersen, and Eggstein: Jour. Exper. Med., 1915, 21, p. 239. Bronfenbrenner: Ibid., 1915, 21, p. 221.

The question whether the ferment exists always preformed in the serum is answered in the affirmative by Bronfenbrenner, but Jobling and his co-workers state that in human serum the ferment exists free only under certain circumstances, such as pregnancy and cancer. Normally it is contained in the leukocytes. In the blood of guinea-pigs and rabbits it exists, however, under normal conditions. But they agree that the ferment normally, tho free in the blood, is prevented from developing its effect by the existence of an antiferment, and such they consider the lipid substances of the blood to be. They state moreover that every substance that is capable of adsorbing lipoids can also set free the ferment of the blood in which it exists.

Bronfenbrenner is of the opinion that the fixation of lipoids under fitting circumstances is perfectly analogous to the fixation of complement and takes place where a specific antigen unites with its specific antibody.

Under normal circumstances the blood ferment in vivo as well as in vitro is inactive, but we have in the anaphylactic shock a clinical picture that, as all workers agree, is due to the intoxication of the organism with toxic split products of albuminous substances resulting from the effect of some tryptic ferment.

The first to state this opinion was Friedberger,<sup>15</sup> who in a series of papers developed the doctrine of anaphylatoxin, but his opinion was that the antigen is split by the complement, after being sensitized by the amboceptor. But it soon became apparent that neither the immune body nor the antigen<sup>16</sup> was necessary for the splitting. Serum was split with kaolin (Keysser and Wassermann), agar (Besredka<sup>17</sup>), or other substances. Jobling and Petersen have made further experiments along this line. They state that the anaphylactic shock comes into existence through the union of antigen and antibody in vivo. The substance resulting is capable of adsorbing the lipoids in the serum, thus setting at liberty the tryptic ferment.

The relation of this ferment to the complement is not as yet clear. They cannot be identical, but it may be possible that the complement is a ferment of other nature and effect, a lipase (Jobling, Petersen, and Eggstein).

<sup>15</sup> Ztschr. f. Immunitätsf., 1912, 12, p. 241; 1913-14, 20, p. 405; 1913, 17, p. 506; 1912, 12, p. 241.

<sup>16</sup> Keysser and Wassermann: Ztschr. f. Hyg. u. Infektionskrankh., 1911, 68, p. 535.

<sup>17</sup> Compt. rend. Soc. de biol., 1911, 71, p. 413.

## IX

After the preceding statements it appears probable that in the blood under normal as well as pathologic circumstances, in vitro as well as in vivo, there takes place a very complicated ensemble between several ferments and antiferments, all of them colloid substances whose degree of dispersity has great influence on their effect. And more, the equilibrium of these substances is a very labile one, so that a small change may give opportunity for the most far-reaching alterations.

So it may be possible that the colloidal changes which take place through the union of antigen and antibody may disturb the equilibrium of the whole system and inactive ferments of any nature may be activated or active ferments put out of action by adsorption to the new formed colloidal union.

If we can produce sufficient experimental support for this hypothesis, it may be found that the colloidal chemical relations are nothing more than an introduction to the real process, or figuratively speaking, the spring that starts the machine. As to the protective ferments of Abderhalden, the argument appears rather complete, but for the genuine processes of immunology we are still far away from this point.

I might venture to suggest the possibility that the pathogenesis of a great many of the infectious diseases may be explained from this point of view; namely, that the living bacteria or protozoa disturb the colloidal equilibrium of the liquids of the organism and so put normal ferments out of action or pathologic ferments into activity. Theoretically such a process appears very probable, but as yet we have no experimental support for it.

## X

But still it may be possible that colloidal processes may be of some direct importance in immunologic phenomena. Bull<sup>18</sup> has discovered that agglutinations may on occasions take place in vivo. He studied rabbits with pneumococcemia. When he injected pneumococcal serum intravenously into these animals, he could immediately afterwards (for 30 seconds) observe an agglutination of pneumococci in the blood. But very soon the agglutinated bacteria were filtered from the blood by liver, spleen, and other organs. Here they were promptly taken up by the phagocytes, more promptly than pneumococci which had not been agglutinated. It may not be improbable that the phagocytosis itself is more related to colloidal phenomena than we think; the ameboid move-

<sup>18</sup> Jour. Exper. Med., 1915, 22, p. 457.

ments of the leukocytes as well as of the ameba are in reality due to local changes in their surface tensions, and it is probable that the action on bacteria and opsonin is such as to make them able to provoke such changes in the surface of the leukocyte when they touch it.

## XI

If, finally, I try to summarize the relations between immunology and colloidal chemistry I shall first venture to assert that we surely shall be at fault if we neglect the colloidal chemical laws in immunologic researches. On the contrary we shall be more right if we concentrate our attention first on those laws and examine how much of the problem they will be able to explain.

But on the other hand it is quite clear that only a small part of immunology will be explained through alternations and adsorptions, the real process in most cases being quite another.

We therefore have always to consider the colloidal chemical part of our immunologic investigations only as preliminary, as a gate to the real process.

In vivo we do not encounter the colloidal chemical processes so pronouncedly as in vitro. The alternations and adsorptions observed play a small part only. What we observe is the effects of the changes in the liquids brought about by these processes. In conclusion I think I may be right in assuming that colloidal chemistry is an important factor in immunology tho but one factor of several. That as isolated method it does not explain anything at all, but as a help to disentangle the complicated processes concerned it may be of immense service.

# STUDIES ON EXPERIMENTAL SCURVY IN GUINEA-PIGS \*

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The earlier literature relating to scurvy, both clinical and experimental, has been so recently and thoroughly reviewed by Holst and Fröhlich<sup>1</sup> and Hart and Lessing<sup>2</sup> that no detailed discussion of it is necessary here.

In 1907 Holst and Fröhlich were able by feeding guinea-pigs on bread, oats, and unpeeled grains to produce a condition which they stated to be in all essentials wholly identical with that found in human scurvy. In their more recent work they produced this disease by feeding a variety of different foods excluding fresh vegetables, the changes being essentially the same as in the first series.

Hart and Lessing<sup>2</sup> produced experimental scurvy in monkeys by feeding them with condensed milk to which had been added cooked rice and dried pig nuts (Erdnüsse). The first symptom noted was bleeding of the gums with later on loosening of the molar teeth unattended by much swelling or ulceration of the gums. Hemorrhages into the skin were not observed, but hemorrhages over swollen knees, ankles, or wrists were often met with, the swelling usually being accompanied with much pain. Spontaneous fractures occurred if life was sufficiently prolonged. The joints were normal.

These authors agree with Fraenkel that hemorrhage is invariably present and that it is not dependent on the existence of fractures but occurs much earlier, before there is any clinical manifestation of the disease. "Die Färbung des Blutergusses verbunden mit deutlicher Schnittenbildung weist darauf hin dass die Blutung schubweis reifolgt." In agreement with Schmorl and Fraenkel the authors believe that the changed bone marrow, "Gerüstmark," is not dependent on hemorrhage, as held by Looser and Jacobsthal. They think that the primary "Krankheitsnoxe," the nature of which is unknown, exerts a constant influence not only on the origin but on the further development of "Gerüstmark" and thus produces the varying picture. They note a great poverty of blood vessels in the fibrous marrow, to which they attribute the disturbances in endochondral ossification. The general disturbances in bone-production are not caused, they think, by the marrow changes, but are due to a general affection of the whole organism, as Schmorl and others have stated. The changes they observed in the periosteum were small hemorrhages without thickening, and larger hemorrhages which resulted in a thickening of the periosteum. Holst and Fröhlich found in guinea-pigs atrophy and fatty degenera-

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<sup>1</sup> Jour. Hyg., 1907, 7, p. 619. Ztschr. f. Hyg. u. Infektionskrankh., 1912, 72, p. 1.

<sup>2</sup> Der Skorbut der kleinen Kinder, 1913.

tion of the muscle fibers and also disintegration into irregular hyaline droplets and irregular staining properties. Hart and Lessing found hydropic degeneration but no fatty degeneration, and in some fibers a deposition of fine granules of calcium. From their experiments on monkeys Hart and Lessing conclude that the skeletal changes produced are identical with those of scurvy but not with those of rickets.

Pappenheimer<sup>3</sup> working with thymectomized rats observed extensive changes in the bones and teeth of a number of animals, not alone of the thymectomized rats, but of controls and stock as well, and from a study of the condition he believes "that the alterations of the skeletal system are not due to the lack of thymus secretion but are a result of spontaneous disease first described by Morpurgo." He found changes in the zone of proliferation of the long bones and ribs, an increase in width of the cartilage and an irregular proliferation of cartilage cells, the presence of osteoid tissue, density of the spongiosa, with narrowing of the marrow cavities and thickening of the compacta; also marked changes in the teeth, defective and irregular calcification of the dentin, and in some cases hyperemia and necrosis of the pulp. In a later publication<sup>4</sup> he describes the production of rickets in rats by the injection of a suspension of bone marrow of a rachitic animal.

The importance of circulatory disturbances in accounting for the alterations found is generally recognized. Morpurgo<sup>5</sup> states that "Ueber die Pathogenese der Krankheit werde ich nur hervorheben, dass die circulatorische Verhältnisse von Grosses Deutung sind." More recently from a clinical study of many cases of human scurvy Hess<sup>6</sup> arrives at the conclusion that hemorrhages in scurvy "are attributable to a disturbance of the vessel walls rather than to a defect in the normal coagulation of the blood." He has not, however, informed us as to the nature of these changes.

Experimental scurvy has been most often produced by diets of grains. The feeding of milk from one animal species to others has been employed less frequently, with results which differ considerably. Keller<sup>7</sup> and Lane-Clayton<sup>8</sup> could observe no difference between mice and rats fed on raw and those fed on cooked cow's milk; Peiper and Eichloff<sup>9</sup> found that dogs fed on raw and sterilized milk developed a fragility of the bone and small hemorrhages around the epiphyseal borders. Rodet,<sup>10</sup> Bruning,<sup>11</sup> and Moro<sup>12</sup> do not describe such changes. Rabbits, according to Moro, cannot live on cow's or woman's milk. Young pigs, as noted from Bruning's observations, appear to be better nourished by raw than by cooked cow's milk.

Bolle<sup>13</sup> was apparently the first to call attention to a condition in guinea-pigs, having many features similar to infantile scurvy, which was produced by feeding milk. Bartenstein,<sup>14</sup> feeding cooked and raw cow's milk to guinea-pigs, observed similar changes. Fröhlich,<sup>1</sup> repeating these experiments in a more

<sup>3</sup> Jour. Exper. Med., 1914, 19, p. 319.

<sup>4</sup> Ibid., 20, p. 477.

<sup>5</sup> Verhandl. d. deutsch. Gesellsch., 1900, 3, p. 40.

<sup>6</sup> Proc. Soc. Exper. Biol. and Med., 1913-14, 2, p. 130. Am. Jour. Dis. Child., 1914, 8, p. 385. Jour. Am. Med. Assn., 1915, 65, p. 1003.

<sup>7</sup> Ztschr. f. diätet. u. physik. Therap., 1904, 7, p. 90.

<sup>8</sup> Jour. Hyg., 1909, 9, p. 233.

<sup>9</sup> Quoted from Funk, Die Vitamine, 1914, p. 87.

<sup>10</sup> Compt. rend. Soc. de biol., 1896, 48, p. 55.

<sup>11</sup> Wien. klin. Rundschau., 1904, 27, p. 481.

<sup>12</sup> München. med. Wchnschr., 1907, 54, p. 2223.

<sup>13</sup> Ztschr. f. diätet. u. physik. Therap., 1902-03, 6, p. 354.

<sup>14</sup> Jahrb. f. Kinderh., 1905, 61, p. 22.

intense study, concluded that raw and cooked milk produced similar conditions, but that cooked milk produced severer lesions. Holst and Fröhlich contended that the lesions produced by feeding grain are different from those caused by feeding milk. Funk<sup>15</sup> prevented the onset of scurvy and loss of weight by feeding guinea-pigs 50 c.c. of uncooked milk daily in addition to the oats diet. After elimination of the caseinogen and other proteins, the milk still retained its antiscorbutic properties. Attempts to isolate this antiscorbutic substance were unsuccessful.

The occurrence some time ago of a peculiar affection in a number of guinea-pigs in the laboratory of St. Luke's Hospital, previously described by Davis and Moore,<sup>16</sup> led to the present studies concerning the effect of milk diets on guinea-pigs. Nine of 26 guinea-pigs in the animal room at that time appeared partially paralyzed, dragging their legs and moving about the cages with difficulty. The muscles of the legs and the tissues, especially around the wrist and knee joints, were swollen and extremely tender on pressure. The animals showed some emaciation. An epidemic disease of some kind was suspected.

On investigation we found that the diet of the affected guinea-pigs, in addition to oats, green vegetables, hay, and water, consisted largely of milk from a cow having experimental streptococcal mastitis. We at once became suspicious of the milk diet, as there had been no such disease before its use. Experiments were designed to ascertain what factors in the diet were responsible. Groups of animals were fed on various combinations of food chiefly in relation to milk or milk constituents to test both the production of the disease and its prevention. These groups will be considered separately.

#### GUINEA-PIGS FED ON PASTEURIZED MARKET MILK

This milk was purchased directly from the dealers and fed to the guinea-pigs within 24 hours after delivery. The milk had been pasteurized for from 25 to 30 minutes at 104 F. The bacterial count at the time of delivery averaged 20,000 to the cubic centimeter. The bedding in the cages was timothy hay. Thirty-two animals were fed on this milk for 18 or more days. Five remained well. The usual history of the others was as follows:

The guinea-pigs for the first 2 or 3 days did not apparently care for milk, but by the 4th day and from then on they drank it with great avidity. They gained in weight and appeared to be in the best of physical condition. At different dates varying from 10 to 29 days the wrist joints became painful to pressure and palpable swelling followed in the course of from 24 to 48 hours. Occa-

<sup>15</sup> Biochem. Jour., 1913, 7, p. 81. Also Die Vitamine, 1914.

<sup>16</sup> Tr. Chicago Path. Soc., 1914, 9, p. 185.

sionally swelling was found with the first signs of tenderness. The joints continued to enlarge until in some instances they were from two to three times their normal size. After 2 or 3 days, the knee joints underwent similar changes while occasionally the ankle, elbow, and shoulder joints became affected in the order named. While the swelling at the wrist joint was at first observed to be more closely confined to the ends of the ulna and radius, that at the knee joint was about the heads of the tibia and fibula. Altho the swelling occasionally spread to encompass the entire joint and even into the tissues of



Fig. 1. Intramuscular (A) and subcutaneous hemorrhage with swollen wrist joint (B) in Guinea-pig 186 fed for 41 days on raw certified milk.

the leg and calf, it was most persistent around the tibia and it was at this location that subsequent changes were most frequent. The calves of the hind legs were frequently swollen and the skin over the affected muscles sometimes turned blue (seen most in white pigs), and underwent the changes commonly observed following bruises. At first the swollen parts sometimes were soft, sometimes firm like a brawny induration. In animals with a sudden and large swelling, the tissues were softer; where the swelling increased more slowly, a firm hard mass was the result. An enlargement of the costochondral junctions resembling a rachitic rosary often was palpable a week after the initial symptoms, and it developed with little noticeable swelling of the surrounding tissues.

At the commencement of symptoms the animals usually appeared to be depressed, became more or less emaciated, and, if the disease progressed, sometimes died. Only 4 guinea-pigs of this series, however, died spontaneously

from the scorbutic condition per se; these died on the 8th, 29th, 34th, and 35th days after the onset of the disease, and on the 26th, 44th, 55th, and 57th days after commencing the milk diet. Two others died of pneumonia, one on the 66th, the other on the 90th day. The latter had swollen joints on the 18th day, the former had no palpable joints, but old hemorrhages were found about the knee joints at autopsy. Twelve animals were killed from the 18th to the 31st day. Seven of these had lesions around the wrist joints. Fourteen after feeding on milk for a long time were placed on a general diet. Five had no palpable lesions altho fed on milk for from 30 to 146 days. A possible explanation of this will be discussed under the changes in weight of the animals.

The average time for the appearance of swellings or hemorrhages around the joints was 19 days. This factor was variable, however, depending to some extent on the weight and the age of the animal, the heavier and older pigs as a general rule not having lesions as soon as the lighter and younger ones. The earliest lesions were observed in 10 days. The longest period for their development was 29 days. In the majority of animals the first joint enlargement was found between the 18th and 22nd days.

The gross alterations varied considerably, depending on the age and the period of feeding. The most constant alteration found at autopsy was hemorrhage. Hemorrhages were most frequent around wrist joints and in the muscles of the hind legs (Fig. 1). They might be few and small. Occasionally the only lesion was a small hemorrhage into the periarticular tissue of the wrists or into the capsule of the anterior tibial tendon of the knee joint. Hemorrhage with accompanying edema, or edema only, was apparently the cause of the primary swelling of the wrist and knee joints. Extensive hemorrhages into the thigh muscles extending around the hip joints were more rarely observed; subcutaneously they were found commonly in the hind limbs, less frequently in the jaw muscles, almost constantly in the bone marrow around the ends of the long bones, and in a few instances within the joint cavity.

At the wrist joints (Fig. 1), the ends of the radius and ulna were enlarged, yellowish white in color, with the consistency of cartilage. At the knee joint, the upper ends of the tibia and fibula had a similar enlargement; more rarely the lower ends of the tibia, fibula, and femur were enlarged. The bones became fragile, often being fractured in manipulation. Fracture occurred generally in the lower third of the tibia and fibula. More commonly we found fractures at the epiphyseal junctions of the radius, and ulna at the wrists, and of the tibia at the knee. Infractures were sometimes found with the periosteum holding the bone in place. The fractures produced various deformities, which sometimes entirely disappeared after healing.

Swelling of the costochondral junctions with or without hemorrhages was occasionally observed, producing a "rachitic rosary" comparable clinically to that of rachitis in human beings (Fig. 2).

In the region of the hemorrhages there occurred an edema, usually limited in extent. Frequently there was an edema around the inguinal and axillary lymph glands with enlargement of the glands, especially the inguinal group. About 75% of the animals had a definite swelling of these glands with occasional hemorrhage into the gland substance.

The spleen was frequently enlarged. In general the viscera possessed no important gross lesions. We found hemorrhage and ulceration of the stomach in one animal and hemorrhages into the adrenals once. Fragility of the lower maxilla with loosening of the molars, which is often observed when feeding guinea-pigs on various grain diets, was not found. The only changes in the gums in this group were the appearance of a submucous hemorrhage at the



Fig. 2. Enlargement of the costochondral junctions (C) in Guinea-pig 186 fed for 41 days on raw certified milk.

base of the lower incisors of 1 pig, and hyperemia of 3 others. The glistening white color of the lower incisors was occasionally replaced by a dull yellow color extending two thirds of the distance from the base to the end. Petechiae into the follicles of the vibrissae of the lips and muzzle were not found grossly or microscopically.

We have previously mentioned that 5 animals, altho fed for from 30 to 146 days on milk, showed no palpable lesions. The animal fed for 146 days weighed 235 gm. at the beginning of the experiment. The other four were not weighed, but were large guinea-pigs approximately over 300 gm. The average weight of those having lesions was 120 gm., the lowest being 80 and the largest 480.

The weight of the guinea-pigs is important as an index of the age. The age of the guinea-pigs when first fed varied between 2 and 5 weeks, and the lesions occurred most frequently in young animals in which the process of bone and tissue building was at its maximum; in but one instance were symptoms of the disease observed in older animals—a guinea-pig weighing 480 gm. which had swollen wrists on the 17th day. That such a diet occasionally produced a somewhat similar, tho less marked, effect on the older animals was again demonstrated in small old healing hemorrhages around the knee in a guinea-pig which had been fed for 66 days on pasteurized milk with no symptoms of the disease during life. More recent experiments have shown that large guinea-pigs are almost as susceptible as small ones.

The time of appearance of the lesions was variable, depending on the age and weight of the animal. In one group averaging 138 gm. in weight, the first lesions were observed on the 26th to the 29th days; in a second group averaging 130 gm., on the 18th day; and in a third group averaging 120 gm., on the 10th to the 13th days. Of the 4 animals which died, 3 weighed 100 gm. and 1 weighed 135 gm. at the beginning of the experiment.

The following specific case is typical of this group of animals:

Guinea-pig 58.—Commenced feeding on pasteurized market milk March 16. Weight 100 gm. On the 15th day both wrists slightly swollen and tender. Weight 205 gm. The wrists rapidly increased in size, and on the next day the knee joints, especially around the heads of the tibia, were noticeably enlarged. The animal died on the 24th day. Weight 150 gm. Necropsy: Periarticular tissues around the wrist joints swollen and filled with blood. Some hemorrhage into the muscles near the joint. Subcutaneous and intramuscular hemorrhage about the knee joints, which were almost twice normal size. Enlargement of the costochondral junctions with no hemorrhage. Inguinal lymph glands swollen and hemorrhagic. Lungs congested, with a small hemorrhage in one lobe. Other organs normal.

#### GUINEA-PIGS FED ON RAW MILK

It was found by Funk, Fröhlich, and others, that raw milk contains more of the vitamins, or antiscorbutic substances, than are contained in pasteurized and cooked milks. Fröhlich states that milk pasteurized at 70 C. for 30 minutes preserves a large but uncertain portion of its antiscorbutic properties, while milk heated for 10 minutes at 98 C. completely loses this property. We therefore fed small guinea-pigs on raw certified milk.

Nine guinea-pigs fed on this milk developed scurvy by the 22nd day. One group of 3, averaging 87 gm. in weight, had lesions on the 10th to the 12th days. Two of these died, one on the 17th, and one on the 19th day. The second group of 4 weighed 90, 125, 140, and 155 gm. respectively. The smallest guinea-pig had lesions on the 10th day, the others on the 11th day. The guinea-pig weighing 125 gm. died on the 23rd day, the largest on the 48th day. The other two were killed. A third set of 2 weighing 160 gm. and 150 gm. respectively, had lesions on the 22nd day. One died on the 28th day.

The lesions were similar to those of the preceding group. All the animals had enlarged wrists and costochondral junctions. Two had hyperemia and one had petechial hemorrhages at the base of the lower incisors. Fragility of the maxillae and loosening of the molars were not observed.

It is apparent from this experiment that a diet of raw milk is not sufficient to prevent experimental scurvy in young guinea-pigs. The protocol of one of the animals follows:

*Guinea-pig 188.*—Commenced feeding on certified milk February 3. Weight 155 gm. After 11 days the wrists were enlarged and painful. Weight 180 gm. The wrists increased in size and the forelegs from elbow to wrists became swollen. At the same time the knees, especially around the tibia, were enlarged and painful. By the 28th day the enlargement of the costochondral junctions was marked and easily palpable. At this time the weight was 160 gm. The swellings of the joints increased in size up to the 31st day, gradually becoming more firm. On the 37th day 2 small hemorrhages were observed at the base of the lower incisors. These disappeared in 2 days. The enlarged joints slowly decreased in size, the swollen portions taking on the appearance of exostoses. Death on the 48th day. Weight 170 gm. Necropsy: Hard appar-

ently bony swellings around the ends of the radius and ulna of both wrists, and at both ends of the tibia and fibula of both legs, more marked at the knee than at the ankles. Yellowish enlargements of the costochondral junctions, with no hemorrhages. Old hemorrhages in the tissues and muscles around the knees. Enlarged inguinal glands. No other changes found.

#### GUINEA-PIGS FED ON BOILED MILK

Eight animals were fed for a sufficient time for the development of scurvy lesions on milk boiled for 10 minutes. Milk subjected to this process has its entire antiscorbutic property destroyed, according to Fröhlich.

Three of the animals were weighed during the experiment. At the start of the experiment their weights were 115, 115, and 190 gm. respectively. The first lesions were observed on the 23rd, 24th, and 24th days respectively.

One of the 8 guinea-pigs (Guinea-pig 45) died from the disease, two were killed accidentally, and one was chloroformed. The remainder were placed on a vegetable diet after 2 months. Two of the animals did not develop palpable lesions altho fed for 66 days on boiled milk. These were large ones in the same group as those fed on pasteurized milk for 2 similar periods with no gross lesions developing.

The shortest period elapsing before the appearance of joint lesions was 13 days, the longest 24 days; the average for the 6 guinea-pigs was 19 days. It is to be noted that the animal weighing 190 gm. showed symptoms of the disease on the same day as the one weighing 115 gm.

An instructive protocol follows:

*Guinea-pig 45.*—Commenced feeding on milk boiled for 10 minutes January 9. Both wrists swollen on the 13th day. These enlarged rapidly and became firm. Death occurred on the 39th day. Necropsy: Lungs congested. Eight to 10 hemorrhages measuring about 1 mm. in diameter on the posterior wall of the stomach. Mesenteric lymph glands swollen and hemorrhagic. Axillary and inguinal glands enlarged. Periarticular tissues of both wrists and of the right elbow contained hemorrhages. The lower ends of the tibia and fibula markedly swollen and hard. Costochondral junctions slightly enlarged. Other tissues and organs normal.

#### GUINEA-PIGS FED ON MILK AND STREPTOCOCCUS BROTH

Since the guinea-pigs in which we first observed the symptoms of scurvy had fed on milk from a cow with experimental streptococcal mastitis,<sup>17</sup> it was thought wise to ascertain whether streptococci added to milk would produce more rapid or severer lesions.

Seven guinea-pigs were therefore fed on pasteurized market milk to which for each guinea-pig, 20 c.c. of a 24-to-48-hour broth culture of a streptococcus were added daily. Three others were fed on milk boiled for 10 minutes to which a like portion of the streptococcus broth was added. Both *Streptococcus hemolyticus* and *Streptococcus viridans* were grown in the broth. These had

<sup>17</sup> Jour. Infect. Dis., 1914, 15, p. 135.

been isolated from patients with tonsillitis, arthritis, endocarditis and various other conditions.

In the first group the earliest lesions appeared on the 12th day, the latest on the 22nd day, average 17 days. In the second group the first appearance was on the 13th day, the latest on the 19th day, average 16 days. The symptoms and lesions were similar in all respects to those previously described. Two of the animals had their throats swabbed daily with streptococcus broth, but we observed no difference in symptoms between these and those not swabbed. One animal died on the 26th day, 5 days after the appearance of the lesions. For an example of this group the reader is referred to the report on Guinea-pig 16 under the discussion of temperature and blood changes.

#### GUINEA-PIGS FED ON STREPTOCOCCUS BROTH

If a streptococcal infection is the sole cause of the disease, as is suggested by the experiments of Morpurgo<sup>5</sup> and Koch,<sup>18</sup> then animals fed exclusively on broth cultures of streptococci possibly should have earlier and severer lesions. Mixed cultures of hemolytic and green-growing streptococci were grown in broth for from 24 to 48 hours and this broth fed daily to the animals.

All of 8 animals died except one. The latter had a broth diet with hay for 21 days, after which it was placed on a diet of mixed vegetables in addition to the broth. Four of the animals died on the 8th and 9th days. Three died on the 19th, 29th, and 33rd days. Two animals lived for a sufficient length of time to have lesions—that is, 29 and 33 days—but in neither animal did we observe any hemorrhages around the joints usually affected or any swelling of the ends of the long bones. In all these animals we found at autopsy a subcutaneous edema with more or less clear fluid in the peritoneal cavity. In general the lymph glands were larger than normal. Congestion of the lungs with some pneumonia was a feature in each instance. The molars were not loosened, nor were there any hemorrhages in the muscles of the jaw. Macroscopical examination revealed no evidence of scurvy. There was, moreover, slight or no increase in weight.

Cultures of the intestinal tract of these animals contained streptococci of the varieties given in the diet. Cultures from the stomach of 2 fed on oats, and from the stomach and various portions of the large and small intestines of 2 fed on milk, and of normal pigs, contained no streptococci.

#### GUINEA-PIGS FED ON WATER AND HAY

Three guinea-pigs were fed on water and hay. Two were fed for 18 days with no consequent scorbutic lesions and then were placed on a vegetable diet. The third died on the 14th day. The only gross pathologic change was a moderate edema of the subcutaneous tissue with marked emaciation. No gross lesions of scurvy were observed.

In a similar experiment by Holst and Fröhlich<sup>1</sup> the animals had a marked universal subcutaneous edema with some ascites. A few small hemorrhages were found in the muscles and subcutaneous tissues but

<sup>18</sup> Centralbl. f. Bakteriöl., R., 1913, 57, p. 250.

microscopically none of the tissue contained any indications of a scorbutic affection; the bone, however, had a pronounced starvation marrow.

#### GUINEA-PIGS FED ON CARROTS, CABBAGE, AND HAY

Seventeen guinea-pigs were fed on carrots, cabbage, and hay for from 33 days to 4 months. They developed normally, with no symptoms of scurvy. This is the usual diet fed to our guinea-pigs, and on it none has ever developed scurvy.

#### GUINEA-PIGS FED ON MILK, VEGETABLES, AND OATS

Five guinea-pigs were fed on milk, carrots, cabbage, lettuce, hay, and oats. All were on the diet a sufficient length of time to have lesions of scurvy.

Two weighing 155 and 165 gm. respectively had no gross lesions after 4 months on such a diet. One guinea-pig weighing 175 gm. had swollen wrists by the 21st day. These went on to exostoses, the animal having mild symptoms and continuing to gain in weight. Two others weighing 90 and 95 gm. had symptoms first appearing on the 41st day. Three others weighing 110, 141, and 160 gm. were fed on milk and carrots for 50 days, with no consequent pathologic changes. It is to be noted that the smallest animals had the lesions.

A protocol of one of the three animals affected follows:

*Guinea-pig 94.*—Commenced feeding on pasteurized milk, vegetables, and oats April 9. Weight 90 gm. On the 41st day both wrists were swollen and painful. Weight 330 gm. During the following 4 days the animal lost 40 gm. The swollen wrists became firmer, and in a week the enlarged ends of the radius and ulna could be distinguished. After 3 weeks these became apparently bony exostoses and persisted for 4 months. The animal regained its former weight by the 67th day, after which it gained at the normal rate, weighing 400 gm. at the end of 4 months.

It is highly probable that those animals having scurvy drank more milk than the ones not so affected. As these experiments were conducted on groups and not on single animals, we have as yet not determined this factor.

At this point it might be well to reconsider the first outbreak of scorbutic symptoms which occurred in the laboratory guinea-pigs and which led to the present studies. As previously stated, 26 guinea-pigs were in the animal room when we first discovered the condition; 9 of these had symptoms of the disease. Eleven small guinea-pigs including the 9 affected had been purchased about 25 days preceding the outbreak. The actual weights of these were not determined, but we judged that they weighed from 150 to 250 gm. Fifteen animals had

been in the room for several months. None of these was affected as far as a physical examination could determine.

The animals were kept in groups in separate cages. Some had been inoculated with questionable tuberculous fluids and other material. The majority, however, were normal. The diet consisted of oats, corn, green vegetables such as lettuce tops, cabbage, and carrot tops from the kitchen, and raw milk from a cow with experimental streptococcal mastitis. All the animals had the same diet. The milk at first contained enormous numbers of streptococci, but had been free from cocci for 10 days before the first animal appeared sick. The milk had been fed to the animals for over 6 weeks. The diet with the exception of the milk had been the ordinary food of the guinea-pigs for several years. As soon as we suspected the milk as the causative factor in the condition, it was removed from the regular dietary, and thereafter no symptoms of scurvy appeared among the guinea-pigs.

Taking under consideration the preceding experiments, we have a possible explanation for the outbreak. We have demonstrated that milk, whether cooked, pasteurized, or raw, will produce clinical scorbutic lesions in young guinea-pigs in periods varying from 13 to 28 days. Secondly, we have demonstrated that when milk is added to the ordinary vegetable diet of guinea-pigs (the latter diet never having produced scurvy), a certain number of the animals will have clinical evidence of scurvy. But the animals which are usually affected are the young growing guinea-pigs, weighing less than 200 gm. As a general observation it can be stated that the younger the animal, the more severe the lesions. The guinea-pigs which had the lesions were all small. None of the large animals were affected, altho they had been having milk for about 6 weeks. The sick animals had been having milk for about 25 days when the symptoms were observed. This is about the length of time after feeding that we found scurvy produced in the experiments. The pathology was identical with that found in the guinea-pigs in our experiments, as is illustrated by the following protocol:

*Guinea-pig 3.*—On May 24 walked with difficulty, hind legs appearing stiff. Knee joints swollen and tender. The knee joints increased in size during the following 5 days, and the right leg could not be straightened.

May 31.—Right wrist joint swollen. Right knee joint aspirated, small amount of blood being obtained. Temperature 101.

May 2 and 3.—Right shoulder joint swollen. All the costochondral junctions enlarged. Right wrist almost normal in size. Both knee joints smaller than before and firm. Chloroformed. Bled from heart and the blood injected into Guinea-pig 8. Culture of heart blood sterile.

Necropsy: Mesenteric, inguinal, and axillary lymph glands swollen. Tissues around right knee joint swollen, containing many hemorrhages. Lymph glands in the inguinal space greatly enlarged. Left knee joint similar in appearance to the right except that there were fewer hemorrhages and less swelling. No pus in the joint cavities; the articular surfaces normal in appearance. Costochondral junctions swollen but no accompanying hemorrhages. Right wrist slightly swollen and much congested. Abdominal organs normal. Cultures from the knee joints sterile.

Cultures from the heart blood from several of these animals were sterile. Of cultures from the joints, one yielded a hemolytic streptococcus, but as the animal in this case was on a streptococcus milk diet we could not rule out a contamination. The others were sterile. Four times blood was aspirated from the heart of a sick guinea-pig and injected into the peritoneal cavities of other guinea-pigs in quantities varying from 1.5 to 5 c.c., but in only one instance did an inoculated animal have similar lesions, and this occurred before we eliminated milk from the general diet. None of the three animals inoculated and kept on an antiscorbutic diet had any symptom of scurvy.

After ascertaining that milk either had something in it which caused scurvy or lacked some essential which prevented the disease, we undertook a series of experiments to learn if possible with which constituent of the milk this property was associated.

#### GUINEA-PIGS FED ON SKIM MILK

If the fat content of milk was responsible, either raising or lowering this content might prevent or increase the symptoms. According to Funk,<sup>19</sup> milk after removal of its fat by centrifugation loses about 50% of its vitamins and allantoin.

Six guinea-pigs were fed on skim milk which had a fat content of 0.1%. One died of pneumonia in 8 days. Two weighing respectively 120 and 110 gm. at the beginning of the experiment did not have lesions after being on the skim-milk diet for 70 days. The others, weighing 90, 100, and 110 gm., had lesions on the 31st, 18th, and 18th days respectively. One died on the 21st day with the following lesions: edema of axillary regions with enlarged congested axillary lymph glands; swelling and congestion of inguinal glands; swellings around both wrists with periarticular hemorrhages; hemorrhages in the muscles of the neck and under the jaw; swelling of costochondral junctions, and petechial hemorrhages in the stomach wall.

#### GUINEA-PIGS FED ON CREAM

Five guinea-pigs weighing from 120 to 160 gm. were fed on cream with a fat content of from 26 to 28%. All lost weight rapidly and died, the periods varying from 3 to 20 days, with no macroscopic

<sup>19</sup> Biochem. Jour., 1913, 7, p. 211.

lesions of scurvy. In every case the large intestine was distended with light mustard-colored semisolid feces. Apparently the digestive apparatus of the guinea-pig could not properly care for such a large amount of fat. The lipolytic enzymes seemed to be deficient. One would expect this in a herbivorous animal which has to digest only small amounts of vegetable fats.

#### GUINEA-PIGS FED ON MILK AND OLIVE OIL

After we had learned that cream was unsatisfactory, a vegetable fat, olive oil, was added to skim milk. The percentage of oil in this mixture was approximately the same as that of fat in cream. The six animals weighing from 100 to 150 gm. lost rapidly in weight and 5 had died by the 5th day, 1 living 11 days. The pathologic findings were similar to those of the guinea-pigs on cream diet. It was conclusively demonstrated that small guinea-pigs would not live on a diet with such high vegetable-fat content.

*Olive-Oil Injections.*—Since the animals did so poorly on cream and olive-oil diets, we considered the possibility of the prevention of the disease by injections of olive oil. Three guinea-pigs averaging from 120 to 140 gm. in weight were fed with pasteurized milk, and 0.5 c.c. of sterile olive oil was injected subcutaneously each day. All had symptoms of scurvy and swollen wrists between the 15th and 17th days.

#### GUINEA-PIGS FED ON LACTOSE WATER AND HAY

Funk found that in the case of animals on a scurvy-producing diet with antiscorbutic treatment an increase in the carbohydrates necessitated a proportional increase in the antiscorbutics to prevent the development of the disease. In other words, when he increased the carbohydrates, he produced a greater susceptibility in the animals toward scurvy. In order to learn whether the sugar in milk alone could produce scurvy 3 guinea-pigs were fed on 15% lactose water and hay. These were small animals weighing 110, 140, and 150 gm. Two died on the 15th and 16th days with congested lungs; the third lived 31 days. These had no pathologic lesions of scurvy but had the lesions of a starvation diet. They gained very little in weight.

#### GUINEA-PIGS FED ON LIME WATER

It has been held by several authors that the rarefaction of bone is due to a lack of lime salts.

Wright<sup>20</sup> believes this is produced by an acid intoxication and states that he found a diminution of alkali in the blood in 7 cases of scurvy. Stoeckner,<sup>21</sup> when feeding rabbits carbonate of lime, found a pronounced apposition of newly formed bones, while when feeding with oats alone he observed rarefaction and defective apposition. Holst and Fröhlich<sup>1</sup> fed 28 guinea-pigs with carbonate of lime in addition to bread, oats, and other grains with neither prevention of scurvy lesions nor diminution in the bone changes.

We fed 3 guinea-pigs with lime water and hay, the water being saturated with calcium carbonate. Two animals died on the 18th day with no evidence of scurvy, but with edema of the subcutaneous tissue and with some fluid in the peritoneal cavity. The third was placed on a vegetable diet on the 14th day. Two other guinea-pigs were fed daily with 1 gm. of calcium lactate in 150 c.c. of milk. The wrist and knee joints were enlarged on the 37th and 41st days, somewhat later than the appearance of lesions in the case of the milk diet alone. The animals both had severe lesions with swelling of both wrists and knees and fractures of a tibia in each instance.

The data in this experiment, altho few, indicate that calcium lactate will not prevent scurvy.

#### CASEIN AND HAY

Casein (10 gm.) in 150 c.c. of water was fed to 3 guinea-pigs. One died of pneumonia in 8 days. The others after 24 days, during which they gained no appreciable amount, and had swelling of the wrists; one died on the 27th day, the other on the 31st. Autopsy disclosed the following lesions:

*Guinea-pig 101.*—Fed on casein, water, and hay. Died on 27th day. Small hemorrhages around wrist joints and over the heads of both tibia. Slight but distinct enlargement of costochondral junction. Hemorrhages about 1 cm. in diameter into masseter muscles of both jaws and at base of lower incisors, and in large intestine. Lower ends of long bones at the wrist slightly swollen. The last two molars on either side were loose. Lungs congested. Spleen enlarged.

Casein, as is seen from the observations on these guinea-pigs, will produce a severer type of scorbutic lesion than we find occurring with any of the milk diets, as is evidenced by the loosening of the molars with hemorrhage into the jaw muscles. Of the different constituents of milk that were tested, casein was the only one which produced scurvy. Further studies along this line are now in progress.

#### CONDENSED MILK

Two guinea-pigs fed on condensed milk mixed with an equal amount of water had swollen wrists on the 15th and 16th days. Both died on the 21st day. The protocol of one follows:

<sup>20</sup> Lancet, 1900, 2, p. 565.

<sup>21</sup> Virchows Arch. f. path. Anat., 1897, 147, p. 430.

*Guinea-pig 120.*—Commenced feeding on condensed milk diluted with an equal portion of water April 27. Weight 130 gm. Wrist swollen on 16th day. Weight 140 gm. Found dead on 21st day. Weight 120 gm. Necropsy: Hemorrhages into muscles around knee and hip joints. Ends of tubular bones of the wrists slightly enlarged. Marked hyperemia of bony portion of costochondral junctions with some increase in size of the junctions and a widened line of ossification. Last molar teeth loose but no hemorrhages in gums. Spleen weighed 320 mg. in comparison with 130 mg for the spleen of a normal guinea-pig of the same weight. Adrenals weighed 100 mg. in comparison with 20 mg. for the adrenals of a normal guinea-pig. Other organs normal.

The adrenals and spleen of Guinea-pig 121 weighed 210 mg. and 350 mg. respectively. In both instances there was great enlargement of these organs. The lesions were severer than those found occurring in the case of other milk diets, if looseness of the molar teeth is taken as a criterion of severity.

#### THYROID EXTRACT, MILK, AND HAY

It was thought possible that the scorbutic condition might be due to some change such as a deficiency in the internal secretions of the body. Four guinea-pigs were therefore fed on milk and every 2nd day given 2 grains of desiccated thyroids. Three died on the 5th, 9th and 11th days respectively with no gross lesions of scurvy; the fourth had swollen wrists on the 11th day and died on the 15th day revealing typical scurvy lesions at necropsy.

#### GUINEA-PIGS FED ON OATS AND HAY, BREAD AND HAY, AND BRAN AND HAY

The studies of numerous authors have proved that certain grains when exclusively fed to guinea-pigs, will produce very severe lesions.

Holst and Fröhlich placed animals weighing between 300 and 600 gm. on diets of oats, rye, wheat, barley, oats, and groats. Death occurred in 30 days on an average with the symptoms of scurvy previously described but with severer lesions, looseness of the molar teeth occurring in all cases and hyperemia with swollen gums in 18%. Some of the guinea-pigs had hemorrhages beneath the mucous membrane; none had ulcerations of the gums.

As controls for the guinea-pigs fed on milk we placed 11 guinea-pigs on oats, hay, and water. They averaged 140 gm. in weight. The first symptom of scurvy appeared as swelling of the wrists within, on an average, 14 days. In one group of 3 the average was 11 days, in a second group of 3, the average was 19 days, a variation similar to that found in the milk-diet tests. Death occurred on an average within 32 days. In every instance looseness of the molar teeth was apparent, accompanied in several by hemorrhages into the gums or into the muscles of the lower jaw. The extent of the lesions is well portrayed in the following protocol:

*Guinea-pig 98.*—Put on oats, hay, and water. Died on 29th day. Necropsy: Hemorrhages around wrists, knees, into muscles of lower jaw, and into gums at base of lower molar teeth. Fractures of lower epiphysis of radius and ulna of left forefoot. Extensive swelling of wrists and knees. Looseness of all

the lower molars and of the last two upper molars of both sides. Enlargement of costochondral junctions with hemorrhage between ribs. The abdominal organs show postmortem changes, the animal having been dead for 2 days before necropsy.

As far as macroscopical examination could determine, these lesions were identical with those produced by milk but more extensive.

Three guinea-pigs were fed on bread, hay, and water. They averaged 140 gm. in weight. Lesions of scurvy appeared on the 25th day. Two died on the 34th and 35th days, respectively; the third, placed on a vegetable diet on the 32nd day, recovered. The lesions were midway in intensity between those of the milk- and those of the oat-fed guinea-pigs.

Three guinea-pigs weighing 130 gm. each were fed on bran, hay, and water. They died about the 31st day, all having looseness of the molar teeth, hemorrhages into the gums and jaw muscles, besides extensive hemorrhages in the fore- and hind-leg muscles. The lesions were more extensive in character than those found with any other diet.

#### GUINEA-PIGS ON MILK AND OATS

In a preceding paragraph it was noted that Funk prevented scurvy in guinea-pigs by adding 50 c.c. of raw milk daily to an oat diet. We fed 3 guinea-pigs, weighing 140, 155, and 170 gm. respectively, with all the pasteurized milk they could consume plus oats. The first lesions were observed on the 19th, 25th, and 26th days, in somewhat the same order as their weights. The symptoms were as severe as those produced on an oats diet alone. However, the animals gained greatly in weight compared with those fed on oats—that is, from 80 gm. on milk to 200 gm. on oats—before the onset of symptoms, and it may be that this factor prevented their early death, all of them living over 2 months on milk and oats diet. We have here added to a food (milk) which produces a mild degree of scurvy, a food (oats) which causes severe symptoms with death and have as a result a condition somewhat midway between those produced by each alone. This may be due to the weight increase, altho it does not follow that substances which maintain body weight are necessarily identical with antiscorbutic substances. Further experiments are being carried out which may offer some conclusion.

#### GUINEA-PIGS FED ON GOATS' MILK

Because of the ease with which a sufficient amount can be obtained, cow's milk has been oftenest used in milk-diet experiments. Whether the condition produced by feeding cow's milk would be representative of those produced by diets of milk from other animals is a question which led us to test goat's milk. Six guinea-pigs weighing from 110 to 145 gm. each were fed on fresh goat's milk, one set for 80 days, a

second for 44 days. The animals developed normally with no clinical symptoms of scurvy. No microscopical study of these animals was made.

#### TEMPERATURE AND LEUKOCYTE COUNT

The temperature of 9 guinea-pigs fed on pasteurized milk or milk with streptococcus broth (3 were in the scorbutic outbreak in the laboratory and 3 were controls) was taken during the experiments. The average temperature of the first series was 102.6 F., of the second 102.8 F., and of the controls 102.8 F. In 2 animals fed on milk and streptococcus broth the temperature was 104.4 F. and 104.6 F. on the first day that lesions were palpable. In other animals the temperatures averaged about 103 F. on the first day of symptoms. With the two exceptions, the highest figure was 103.8 F. It is apparent that experimental scurvy is a nonfebrile disease in the majority of affected animals.

Gulland and Goodall<sup>22</sup> found the leukocytes of guinea-pigs to be 9,170 to the cubic centimeter and they quote Burnett as finding the number to be 9,000 to the cubic centimeter. Our observations on 5 guinea-pigs with scurvy gave us the average leukocytic count as 8,000, with high and low counts of 9,800 and 7,200. The number of leukocytes did not increase much with the appearance of symptoms, the average before lesions developed being 7,500 and afterwards, 8,200. In a sixth guinea-pig the count was 8,500 white cells to the cubic centimeter before lesions appeared, 10,000 the day swollen joints were palpated, and 12,800 three days later when the animal was killed. The average white-cell count of 3 control guinea-pigs was 8,200. Experimental scurvy, therefore, produces no appreciable leukocytosis in the majority of affected animals. The following protocol is typical of the temperature and of the leukocytic count:

*Guinea-pig 16.*—Commenced feeding on milk and streptococcus broth, July 23. Throat swabbed with streptococcus broth daily. July 27, T. 102.5; July 29, T. 102.6; July 30, T. 103; July 31, T. 102.4, white-cell count 8,000; August 1, T. 101.6; August 4, T. 103; August 5, T. 102.1, white-cell count 8,600, both wrist joints swollen; August 6, T. 103, white-cell count 8,500, right wrist greatly swollen; August 8, T. 103.4, white-cell count 9,500; August 11, T. 102.2, white cells 8,200; August 13, T. 102.6, right knee swollen in addition to wrists; August 15, T. 102.6, white cells 6,400; August 18, T. 103, white cells 5,500, both knees and wrists swollen. Placed on vegetable diet. September 12, chloroformed. Necropsy: Wrist joints swollen and ankylosed. Knee joints swollen and firm with old hemorrhages in periarticular tissues. All lymph glands swollen. Lungs contained areas of congestion. Other organs normal.

<sup>22</sup> The Blood, 1912.

## MICROSCOPICAL PATHOLOGY

In an earlier study by Le Count and Jackson<sup>23</sup> of the histologic changes in the bones and joints in a disease of guinea-pigs resembling scurvy and caused by feeding milk, chiefly late changes were considered. As a careful consideration of the primary alterations in this condition has generally been neglected, it seemed that a systematic study of them in conjunction with that of the later changes might be of value. In the present work some of the animals previously studied are included.

Tissues from 25 guinea-pigs were examined microscopically and those from 19 were selected with reference to securing a series as regards the periods of time elapsing between the beginning of feeding and the death of the animal, the shortest period being 9, the longest 69, days. The duration of the disease in the remaining 6 is not known exactly. Six of the 19 guinea-pigs were killed within the first 3 weeks after feeding was begun and before there were any clinical manifestations of the disease; 3 guinea-pigs were killed 21 days after the beginning of the experiment, or at about the time that symptoms first appeared in the milk-fed animals. All the animals except 3 from which tissues were examined microscopically, were fed on milk and hay without green vegetables, 2 were fed on oats, hay, and water, and 1 on casein, hay, and water.

The parts selected for examination were knee joints from 15 guinea-pigs, ribs from 11, the lower jaw from 6, wrist joints from 4, and the elbow joint from 1. Sections of the skin were also examined in a few instances. The material was fixed in Zenker's fluid and imbedded in paraffin. Serial sections were made from portions of some of the joints. Sections were stained by the usual methods, hematoxylin and eosin, phosphotungstic acid hematoxylin, and by the Wright and Giemsa methods. Wright's stain was much used because it gave excellent results with the bone marrow and also because it requires less manipulation of the sections, a distinct advantage in staining sections containing bone, which is easily detached from the slide.

Changes were found in sections from all but 2 of the animals examined. Hemorrhage was one of the earliest as well as most constant alterations observed. More or less necrosis and small infarct-like lesions were also present in tissues from some of the animals fed for a short period of time. Protocols and descriptions of the histologic changes found in 3 such animals follow:

*Guinea-pig 182.*—Commenced feeding on pasteurized milk January 28. Weight 140 gm. February 6, weight 210 gm. Killed. Hemorrhage into the muscles of the left thigh and below the left knee, otherwise no gross changes.

In some sections of the knee joint there was a necrotic region from 2 to 3 mm. in its longest dimension, which was parallel with the epiphyseal cartilage in the lower end of the diaphysis of the femur. In all the sections of this joint examined there was more or less change in the bone marrow and bone trabeculae adjacent to the epiphyseal cartilage, the marrow being more affected.

<sup>23</sup> Tr. Chicago Path. Soc., 1914, 9, p. 189.

Small hemorrhages; fragmentation of nuclei, and lessened staining properties. Cartilage unchanged. In some sections there was a much distended vein, or sinus, 5 mm. above the epiphysis, and at about the middle point of the shaft there appeared to be a thrombosis of a large vein. Numerous small hemorrhages in the periosteum covering the posterior surface of the femur and a rather large hemorrhage about the large vessels posteriorly which had extended for a considerable distance along some of the intramuscular septa. The place in the vessel wall from which this hemorrhage took place was not found. A less marked but similar condition was present in the upper end of the tibia.

*Guinea-pig 163.*—January 28, began feeding on milk and hay without green vegetables. Weight 145 gm. February 7, weight 175 gm. Killed. Small hemorrhage over the patella of the left knee, otherwise no gross changes. A large number of sections from one knee joint examined. Numerous small hemorrhages and one of considerable size in the tissues outside of the joint posteriorly, all of these being in the immediate neighborhood of veins or capillaries. The largest hemorrhage was about a vein of rather large size, in the wall of which were definite changes. Marked thinning of the wall (Fig. 3), as tho the wall as a whole had partially melted away leaving few traces. In this portion of the wall were many small round bodies resembling cocci (Fig. 3), which stained a deep blue by the Wright and Giemsa methods. These bodies were also present in the lumen of the vessel and in the inner layers of the more normal portions of the wall. In the lumen adjacent to the thinned portion of the vessel wall was a small amount of a finely granular pink-stained material. At one point in the lining of the joint cavity just beyond the cartilage was a minute region of surface necrosis (Fig. 4) from which a small triangular mass of eosin-stained material resembling fibrin extended into the cavity. In the midst of this mass were a number of the coccus-like bodies. No inflammatory reaction in or about these regions of hemorrhage and necrosis, with the exception of many eosinophile cells, both mononuclear and polymorphonuclear, which were particularly numerous around blood vessels and in the bone marrow. Changes in some of the muscle fibers, particularly those attached to the periosteum. Portions of the fibers (Fig. 5) partially or completely disorganized and replaced by a small amount of finely granular material.

*Guinea-pig 154.*—September 3, began feeding on milk and hay without green food. Weight 120 gm. September 17, weight 150 gm. Killed. No gross changes present in any of the organs. Sections of both knee joints, the lower jaw, and two ribs were examined. A well-defined region resembling an infarct (Fig. 6) in about the middle portion of the shaft of the tibia and just anterior to the greatly dilated central vein draining the upper part of the bone. A series of sections through only a part of the lesion was obtained and in these no occluded vessel was found. There were hemorrhages and much fibrin present in this region but no necrosis. The vein in the neighborhood of this lesion occupied two-thirds of the diameter of the shaft at this level. Hemorrhages and a small region of necrosis occurred in the bone marrow and bone at the lower ends of the diaphysis of the femur near the periphery posteriorly. In the other knee joint there were also small hemorrhages in the bone marrow close to the epiphysis and about the veins in the popliteal space. An infarct-like lesion similar to that in the tibia was present in the pulp of one of the incisor teeth. In sections of one of the ribs there was hemorrhage into the marrow near the costochondral junction with a small amount of necrosis at the periphery.



Fig. 3. A portion of the wall of a vein in the popliteal space of Guinea-pig 183. The figures indicate (1) a thinned portion of the wall, (2) red blood corpuscles outside the vessel wall, and (3) bacteria.

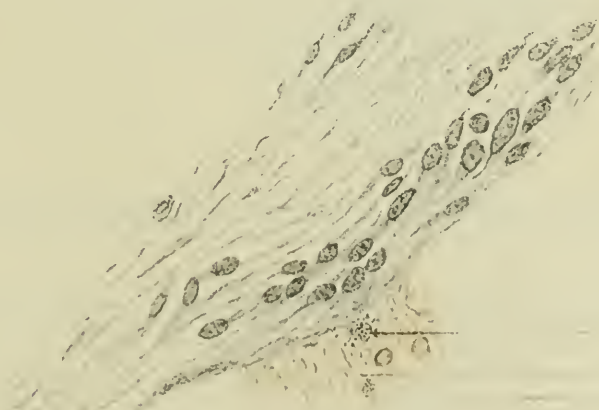


Fig. 4. A portion of the lining of the joint cavity of Guinea-pig 183. The figures indicate (1) a small mass of fibrin-like material, and (2) a clump of bacteria.



Fig. 5. A portion of the muscle and periosteum from a section of the knee joint of Guinea-pig 154. The figures indicate (1) the periosteum, (2) normal muscle fibers, (3) degenerated muscle fiber, and (4) a capillary.

In charting the lesions observed in the tissues from all these animals it was discovered that hemorrhage was present in all the guinea-pigs in which there were any alterations. They were widely distributed, being found in the muscles, bone marrow, periosteum, tooth pulp, and occasionally in the skin.

Hemorrhages occurred in the bone marrow at many points, but they were especially frequent at the end of the diaphyses of long bones, near the costo-

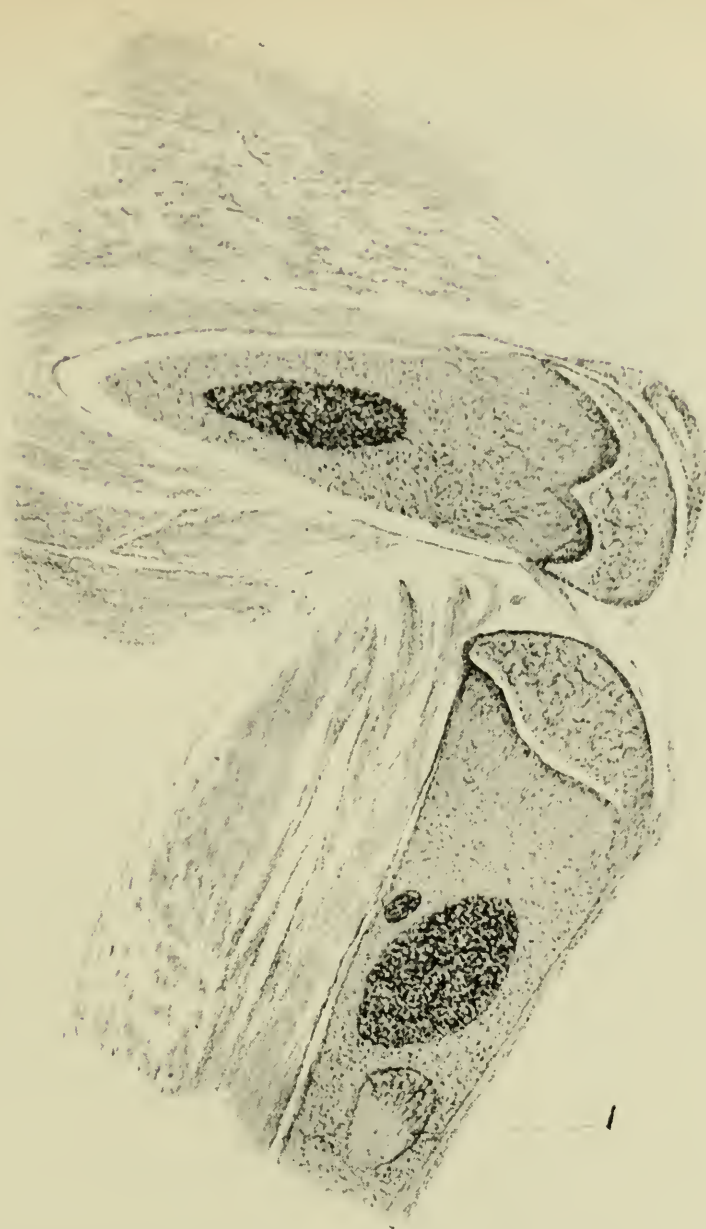


Fig. 6. A section through the knee joint of Guinea-pig 154. The figure (1) indicates a small infarct-like lesion in the bone marrow.

chondral junctions of the ribs, about blood vessels near the point where they pass through the compact bone of the shaft of long bones, and in the bone marrow of the lower jaw near the teeth, and also in the tooth pulp. Outside the bone they were present in the muscles of the leg and thigh, but particularly about the large vessels in the popliteal space, in the muscles over the ribs close to the costochondral junctions, and in the muscles of the lower jaw, particularly about the attachment of the masseter muscle to the posterior portion of the inferior maxilla. When the disease was of long standing, there were deposits of pigment, some of it contained in large endothelial cells, with the surrounding tissues more or less changed, marking the site of the old hemorrhage.

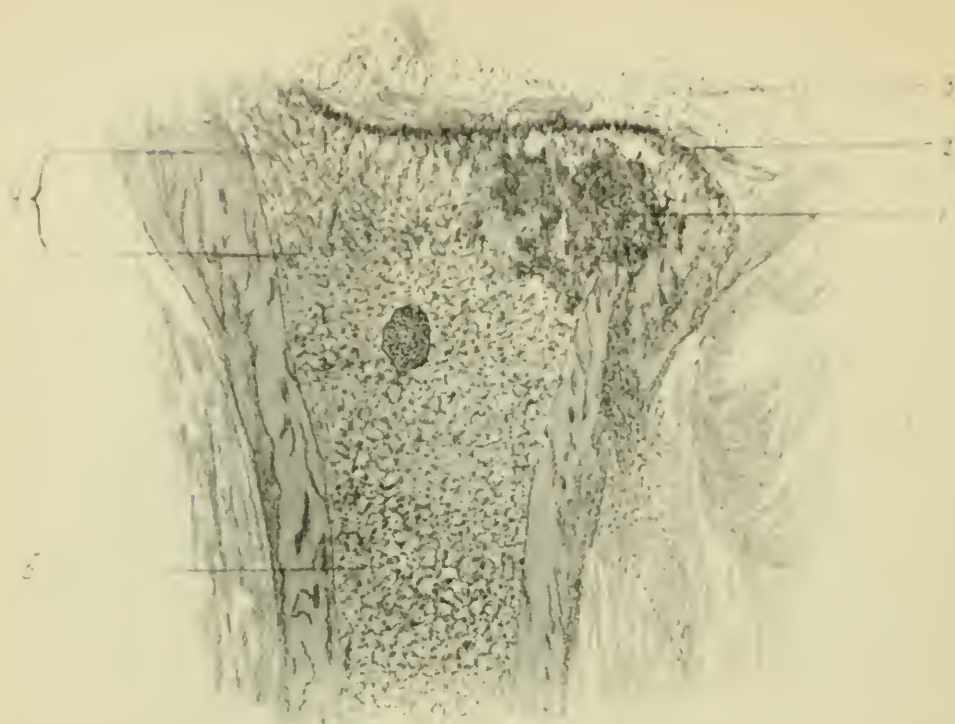


Fig. 7. A section through the lower end of the femur of Guinea-pig 7. The figures indicate (1) a region of necrosis, (2) epiphyseal cartilage, (3) the marrow of the epiphysis, (4) a region of osteoid tissue, and (5) the marrow of the shaft.

Hemorrhages varied as to size and character. There was often a uniform scattering of red corpuscles through rather large regions of bone marrow, this being usually associated with a decrease in the lymphoid elements. Hemorrhage of this kind was frequently found in the marrow of the wrist bones and in the tooth pulp. Small and well-circumscribed hemorrhages occurred early in the disease and in the milder forms. These as well as some of the larger hemorrhages when examined early in the disease were found associated with definite alterations in the walls of veins. In general the large hemorrhages occurred in the severe and later forms of scurvy and appeared to take place gradually. In some specimens examined the popliteal space was filled with blood and the muscles and spaces between thoroughly infiltrated with blood. Infarction of the upper end of the tibia or of the lower end of the femur was accompanied by large hemorrhages at the epiphyseal junctions, and usually also at about the middle point of the shaft (Figs. 7 and 8) both in the marrow and in the tissues outside the bone where the vessels pierce the compacta. With marked changes in the teeth there was often great dilatation of the veins of the pulp attended by more or less hemorrhage into the pulp (Fig. 9) and also into portions of the alveolar process immediately in contact with the teeth. Holst and Fröhlich observed petechiae in the skin, which with one exception were found in the follicles of the vibrissae. Altho looked for with considerable care in animals having severe lesions elsewhere, no hemorrhages in this location were found. In a few guinea-pigs, however, especially those subjected to experiment for the second time, we observed bleeding into the inner layers of the skin. These lesions varied in size from that of pinhead size to those 1.5 cm. in diameter. Microscopically the hemorrhage was just external to the muscular layer. In

these regions in Guinea-pig 299 there were numerous large endothelioid cells, many of them multinucleated, but no polymorphonuclear leukocytes.

The axillary, inguinal, and popliteal lymph glands were enlarged and often hemorrhagic. In sections it appeared that the hemorrhage was entirely into the lymph tissues, and in some cases practically all of the red blood corpuscles had been taken up by, or were attached to, large lymphocytes or endothelial cells. In more advanced stages of the disease these cells contained pigment. In the adipose tissue about the popliteal lymph gland from one of the animals a considerable number of multinucleated giant cells were found.

Lesions having the shape, location, and characteristics of infarcts were found in the ends of the diaphyses of long bones. In one of the animals fed on oats, hay, and water (166) there was complete infarction of the upper end of the tibia. As previously mentioned, we also observed small well-defined ovoid lesions in the bone marrow (Fig. 6) and in the pulp of the teeth which resembled infarcts. There was some hemorrhage with fibrin and edema of the tissue, but no occluded vessels were found. In a few instances clots with fibrin-formation, disintegration of nuclei, and beginning organization were present in the large central veins of the long bones; also rather frequently in this location and in the tooth pulp (Fig. 9) greatly distended veins were seen, about which there was more or less hemorrhage.

Guinea-pig 165 illustrates both the severity of the changes that developed in guinea-pigs fed on oats and water and their similarity to those produced by feeding milk.

*Guinea-pig 165.*—August 22, began feeding on oats, hay, and water without green vegetables. Weight 130 gm.

Sept. 9.—Weight 170 gm. Congestion of the gums at the base of the incisor teeth.

Sept. 14.—Wrists swollen and painful, also the knees. Gums congested as on the 9th. Weight 180 gm. From this time on there were a gradual loss in weight, and increased swelling and painfulness of the joints and muscles until on the 22nd the animal was unable to walk.

Sept. 29.—Died. Necropsy: Lungs slightly congested. Spleen about twice normal size. Lymph glands large and congested. Hemorrhages into the muscles of the thigh. Both wrists markedly swollen and red. The heads of both tibiae enlarged, with surrounding hemorrhages. Costochondral junctions greatly enlarged and the last molar teeth slightly loosened.

In sections of the knee joint, huge hemorrhages could be seen with the unaided eye in the tissues about the large vessels posteriorly and in and between the muscles of the leg and thigh, also lightly stained regions, one of which comprised a zone 2 mm. wide adjacent to the epiphysis in the bone marrow of the lower portion of the diaphysis of the femur. Most conspicuous were the hemorrhages present in all parts of the sections but particularly in the popliteal

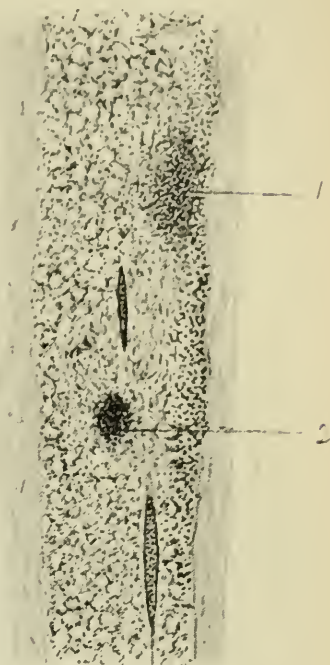


Fig. 8. A section through the shaft of the femur of Guinea-pig 7. The figures indicate (1) a region of hemorrhage surrounded by osteoid tissue and (2) a region of hemorrhage surrounded by marrow.



Fig. 9. A section through a molar tooth of Guinea-pig 165. The figures indicate (1) an alveolar process, (2) dentin, (3) pulp, and (4) a hemorrhage.

space, in the vicinity of the lower epiphysis of the femur, and in the periosteum. The largest hemorrhages were outside the bone.

There was a decided decrease in the lymphoid cells in the lightly stained portions of the bone marrow (Gerustmark) and in their place were many spindle-shaped cells, among which were scattered red blood corpuscles. A golden-brown pigment was present in some of these cells, especially near the epiphyseal cartilage, where the other changes just mentioned were most pronounced. At either margin of the bone and between the zone of osteoid tissue and the epiphyseal cartilage there were narrow strips of necrosis extending inward along the cartilage about 1 mm. Opposite these regions of necrosis were small regions of rarefaction of the marrow in the epiphysis similar to that found in the shaft. In places there was an absence of the compacta for a distance of a few millimeters above the epiphyseal cartilage. The periosteum covering the lower portion of the femur was greatly thickened and contained many small hemorrhages.

Marked changes occurred in the tissues immediately about the joint, particularly in the regions of large hemorrhages posteriorly. There was complete disappearance of muscle fibers from areas of considerable size in the immediate vicinity of large hemorrhages, and in other places of a few or single fibers, adjoining fibers being little or not at all changed. These were replaced by spindle-shaped connective-tissue cells, some containing a golden-brown pigment, and in some places by a granular eosin-staining material. In the less altered portions of the muscles there was a great increase in the number of nuclei. Sometimes 3, 4, or 5 small deeply stained nuclei lay close together in a row. In places large irregular shaped masses of chromatin were found in the muscle fibers.

In sections of the lower jaw hemorrhages were numerous in the bone marrow, periosteum, and tooth pulp. The hemorrhages in the periosteum were especially noticeable in the portions covering the alveolar process. There was great dilatation of some of the vessels in the pulp of the molar teeth at about their middle with considerable hemorrhage about them (Fig. 9).

Descriptions of the alterations found in tissues from Guinea-pigs 16 and 3, the protocols for which have been given, furnish very good examples of the appearances obtained in the later stages of the process when healing is in progress, or, as in some parts, complete.

In Guinea-pig 16 the line of junction between the bone and costal cartilage instead of being well-defined was irregular (Fig. 10) because of small trabeculae of cartilage which projected towards the marrow of the bone for a short distance. There were many of these as wide as 2 or 3 cartilage cells. They extended out to about the same distance from the end of the cartilage and for almost the entire width of the shaft. A thin layer of necrotic tissue covered them; in other words, there had been a necrosis of the costal cartilage. Farther towards the shaft of the bone was a narrow zone of loosely aggregated fibroblasts—osteoid tissue—containing irregular masses of cartilage and bone. This was two or three times the width of the trabeculae of cartilage previously mentioned. The necrosis was always most marked at the edges and least marked at the middle, therefore the sections cut from the surface possessed the largest amounts of necrosis. In the periosteum and surrounding muscles were hemorrhages of considerable size. The changes in sections of the knee joint were similar to those described in the rib. In the wrist joint, however, the distal end

of the diaphysis of the radius was occupied by a very mature callus—heavy spongy bone—with large amounts of pigment in the marrow.

In Guinea-pig 3 in sections of a rib on either side masses of cartilage close to the periosteum had replaced the break caused presumably by necrosis, and spongy bone formed the middle of the callus. The bone marrow in this region was fibrous with almost no lymphocytes present. The tissue surrounding this portion of the rib contained old hemorrhages and many cells contained a golden-brown pigment. Healing had reached a somewhat advanced stage in the muscles and other tissues surrounding the knee joint. The regions of hemorrhage, especially about the large vessels posteriorly, were quite thoroughly grown through with large irregular stellate and spindle-shaped cells with large nuclei containing one or more nucleoli. Some of these cells stained very faintly. Small round cells were also present but were less numerous. There was more or less infiltration in these cells about the blood vessels (Fig. 11) even in the more normal parts of the section. The large cells also filled in small defects in the muscles at various points. They frequently contained pigment. A noticeable feature was the swollen endothelium of some of the blood vessels with, seemingly at least, an increase in the number of cells. The endothelium in places in the larger vessels had a vacuolated appearance.

In the earliest lesions there was no change in the tissues at the site of hemorrhage. Necrosis was not a prominent feature until after the third week in milk-fed guinea-pigs. However, in the earliest specimens studied there were some fragmentation of nuclei, loss of staining properties of the cells, and formation of a fine fibrin network in places in the bone marrow of the ends of the diaphyses of some of the long bones with occasional small hemorrhages in some, accompanied by hemorrhages of considerable size in others. Definite small regions of necrosis were also occasionally present in this location. Changes were noted in some of the veins outside the bone in the 9-, 10-, and 14-day guinea-pigs. The walls of veins in the immediate vicinity of hemorrhages were sometimes thinned in certain places, with separation of the parts by red blood corpuscles and occasionally a complete break. The appearance was that of a complete disappearance or melting away of parts leaving scarcely a trace. There was a small amount of nuclear fragmentation, and large numbers of coccus-like bodies were present in the wall and in the lumen.

From a histological study of this disease it appears that the changes were due to some agent of a mildly destructive nature apparently acting chiefly on the circulatory apparatus to cause the hemorrhages so frequently encountered in this condition, but likewise causing alterations in the muscle fibers, bone, and bone marrow. In the latter stages especially it was impossible to decide how much destruction was due to the primary cause and how much was secondary to hemorrhage, but a study of the early stages makes it evident that at least a part of the

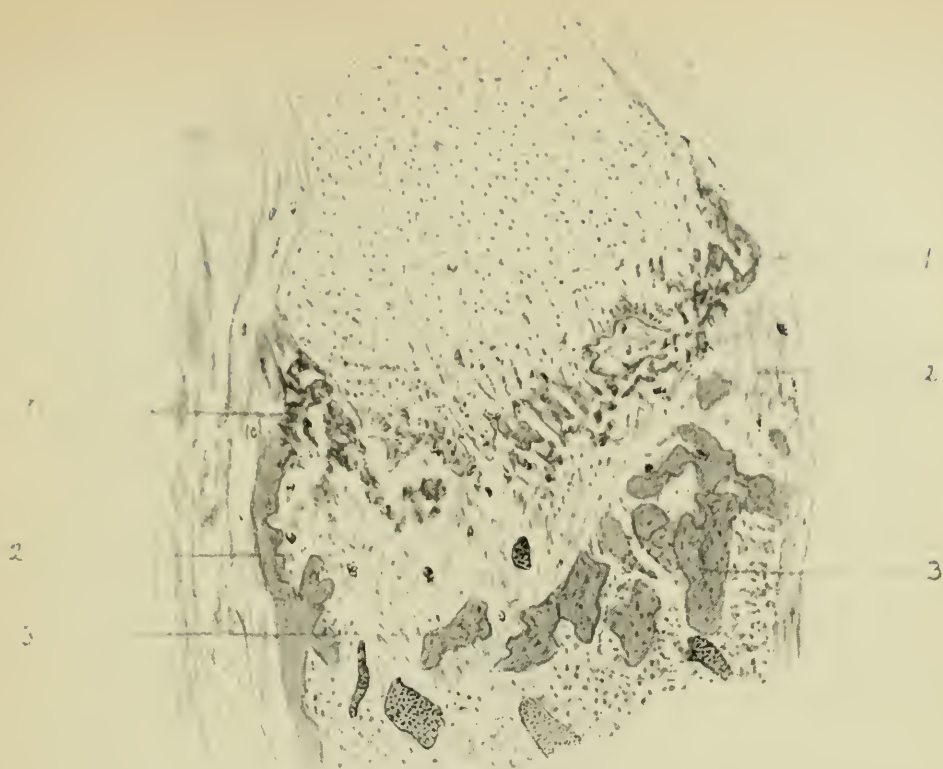


Fig. 10. A section through the rib of Guinea-pig 16. The figures indicate (1) a zone of necrosis near the cartilage, (2) a zone of osteoid tissue, and (3) irregular trabeculae of bone.



Fig. 11. Infiltration about a small blood vessel in the muscles of the leg in Guinea-pig 3.

changes in the muscles and bones were not the result of hemorrhage. Certain features of the process seem to indicate that we may have been dealing with a mild infection.<sup>23</sup> It is true that a marked inflammatory reaction was lacking, but it is a localized process, there being certain favorite points of attack, some and not all points being affected at the same time, except when the disease is severe and prolonged. Altho the most prominent feature was the bone changes, the disturbance did not seem to be with the mechanism of bone-formation as a whole, but disturbances at certain definite points interfered with normal



Fig. 12. A section through the middle of a rib of Guinea-pig 11. The figures indicate (1) a small region of necrosis, (2) blood vessels, (3) bone trabeculae, and (4) cartilage.

processes there. For example, marked alterations might occur in one bone of the knee without the others' being similarly affected or some and not all the ribs might undergo changes. Moreover, changes of the same character occurred outside the bones in the muscles and other soft tissues. Naturally the attention was not so forcibly attracted to alterations in these tissues as the results were not so momentous for the growing organism. The frequent occurrence in these lesions of bodies having the staining properties and other characteristics of bacteria also strengthens the impression. It likewise seems

<sup>23</sup> Jackson and Moody: Jour. Infect. Dis., 1916, 19, p. 526.

probable that the hemorrhages, so conspicuous in this condition, might have been due to localized injuries to the vessel walls and that the agent causing the disturbance in the blood vessels might also have been responsible at least in part for the changes in the ends of the diaphyses of long bones, in the muscles, and elsewhere, aside from rather large numbers of eosinophile cells in these regions where there was no inflammatory reaction in the tissues.

Necrosis occurred at the costochondral junctions of the ribs and at the ends of the diaphyses of long bones affecting chiefly the bone and bone marrow, to a less extent the cartilage, and was most marked at the periphery, where the compact bone was sometimes completely destroyed for a short distance. As might be expected fractures at these points were rather frequent. The margins of the cartilages were uneven, the arrangement of the cartilage cells irregular, and, especially at the costochondral junctions, the cartilage bulged towards the marrow cavity. In the guinea-pigs fed on oats and hay there was almost complete necrosis of the pulp of the incisor teeth, also more or less necrosis in the pulp of the molars.

The appearances observed in the process of healing naturally depended largely on the extent of the earlier changes and the duration of the disease. If the alterations had been slight there might simply be changes in the character of the bone marrow near the costochondral and epiphyseal junctions with slight increase in width at these points and evidences of old hemorrhages both within and without the bones. Following the marked early changes, large hemorrhages, necroses, and fractures, there was more or less deformity, with enlargement of wrist and knee joints especially, and an increase in width of the costochondral junctions. The reparative process in bone in this disease was such as would normally take place after an injury of like severity and of slight inflammatory character, by the formation of osteoid, cartilagenous, and bony callus. One observed in the region of the epiphyseal cartilage a fibroid marrow, marked irregularity of the diaphyseal margin of the epiphyseal cartilage (Fig. 13), and at the periphery osteoid, cartilagenous, or bony callus (Figs. 12, 13, and 14) according to the stage in the process.

Changes in the muscle fibers are of interest. These alterations were noticed particularly in the muscles of the leg and thigh. Early in the disease and also in the later moderately severe cases a very small or considerable portion of the fiber might be completely disintegrated while other parts and adjacent fibers appeared unchanged.

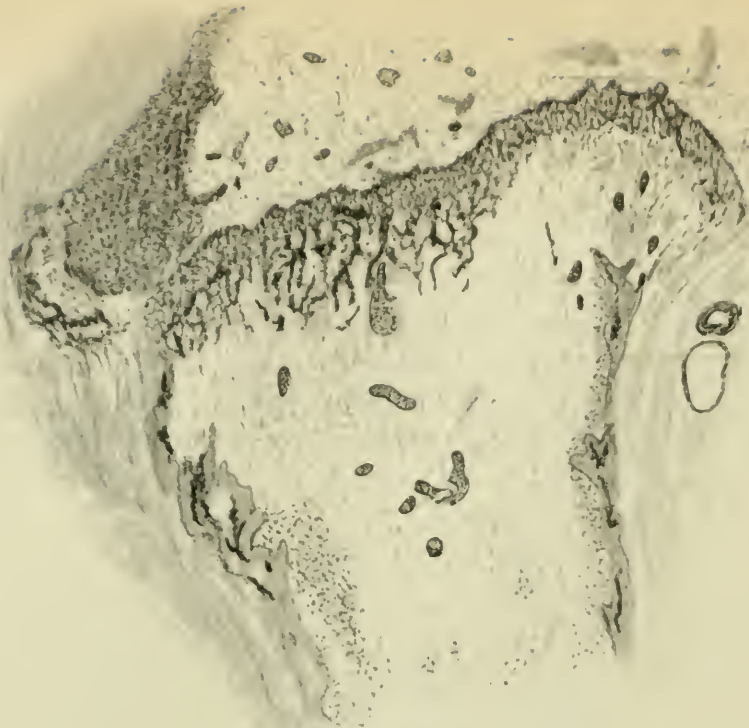


Fig. 13. A section through the upper end of the tibia of Guinea-pig 1. The figures indicate (1) bone marrow of the epiphysis, (2) trabeculae of bone, (3) epiphyseal cartilage, and (4) osteoid tissue.

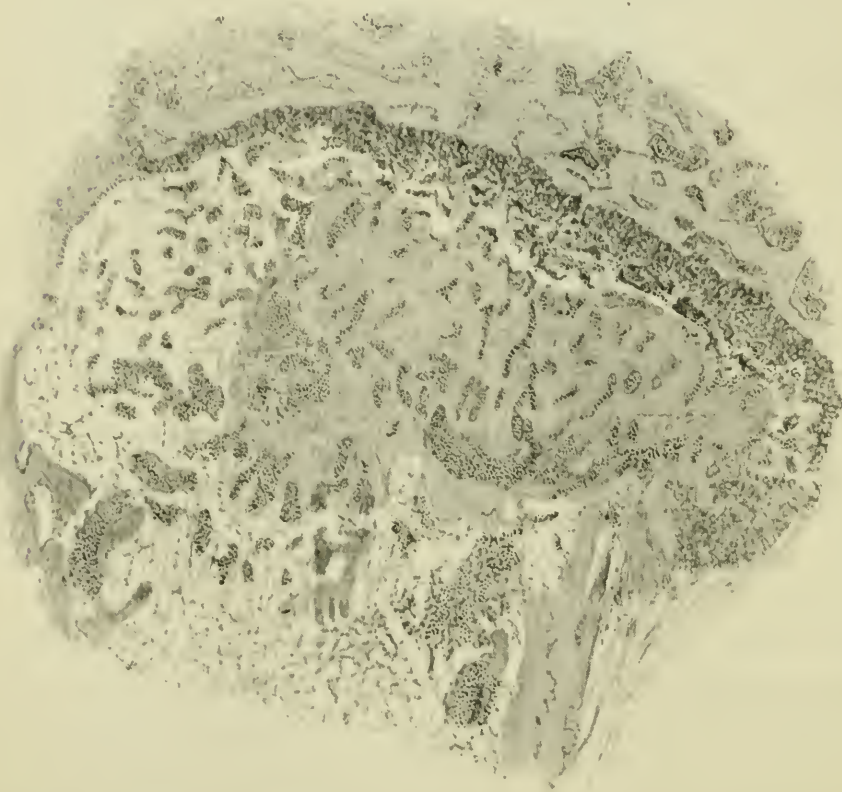


Fig. 14. A section through the upper end of the tibia of Guinea-pig 11. The figures indicate (1) marrow of the epiphysis, (2) trabeculae of bone, (3) epiphyseal cartilage, (4) callus made up of dense spongy bone, and (5) marrow of the shaft.

The degenerative process here was of the same character as that in the blood vessels and appeared very frequently to be entirely independent of hemorrhage. Lesions in the muscles appeared as early in the disease as hemorrhages and their severity varied with that of the lesions elsewhere.

#### SUMMARY

Experimental scurvy was produced in guinea-pigs by diets of pasteurized, raw, boiled, skimmed, and condensed milk, streptococcus broth and milk, milk and green vegetables, thyroid extract and milk, casein and water, oats, bread, and bran. The addition of calcium lactate to milk or the injection of calcium lactate into guinea-pigs on a milk diet did not prevent scurvy. A cream diet, and a diet of olive oil added to milk, produced a "fat constipation" with early death. Daily injections of olive oil into animals on a milk diet had no antiscorbutic effect.

Mixed broth cultures of *Streptococcus viridans* and *Streptococcus hemolyticus*, water, lactose water, and lime water did not produce scurvy.

In a series of 6 guinea-pigs fed on goat's milk for over 40 days, no symptoms of scurvy developed.

In guinea-pigs fed on milk, the clinical symptoms, in brief, were preliminary loss of weight, swelling of the wrist and knee joints, occasionally of the costochondral junctions, ankle, and elbow joints, and occasionally hyperemia of the gums with dullness of the lower incisors. Fractures of the long bones near the epiphyseal ends were common; fragility of the bones was more or less marked. Exostoses and deformities were frequent, especially when the milk diet was continued for several weeks.

The average time for the onset of symptoms with pasteurized milk was 19 days. With other milk diets this varied from 11 to 19 days. The earliest lesion was observed on the 10th, the latest on the 29th day.

The disease was afebrile and produced no great increase in leukocytes.

The chief pathologic lesions noted post mortem were hemorrhages, which were found in the muscles, bone marrow, more frequently at the ends of diaphyses, tooth pulp, costochondral junctions, and occasionally in the skin and lymph glands; enlargements of the ends of the long bones, especially the lower ends of the radius and ulna, the upper end of the tibia, and the costochondral junctions; and swollen lymph

glands, especially the inguinal and axillary. The enlargement of the bones was often accompanied by fractures near the epiphyseal junctions.

Microscopically the earliest lesions observed were slight amounts of necrosis and hemorrhage in which coccus-like bodies were frequently demonstrated, the only evidences of inflammation being slight fibrin-formation and the presence in the surrounding tissues of large numbers of mononuclear and polymorphonuclear eosinophile cells. Small infarct-like lesions were also found early in the process. The later changes were those customarily found and previously described in experimental scurvy in guinea-pigs.

Cultures of the heart blood from guinea-pigs with scurvy from milk diet were sterile, and passage of blood from these animals to normal animals did not produce the disease.

# BACTERIOLOGIC STUDIES ON EXPERIMENTAL SCURVY IN GUINEA-PIGS \*

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Various authors investigating diseases in laboratory animals affecting chiefly the skeletal system have ascribed to them an infectious origin.

Murpurgo<sup>1</sup> studied a disease occurring among white rats which produced most marked changes in the bones. Microscopically there was a rarefaction of the bone substance, a broad zone of fibrous marrow replacing the normal bone and bone marrow with pigment, especially in the neighborhood of blood vessels, and in some cases islands of cartilage in the shaft of the bone, which the author was at a loss to account for unless they bore some relation to callus-formation following infarction. He says: "Ueber die Aetologie kann, nach den erfolgreich durch vier Reihen von Ratten mittels den von jeder Reihe rein wieder gewonnenen Diplococcen ausgefeuterten künstlichen Infektionsversuchen wohl kein Zweifel obwalten." From the spinal cord, and later from the organs and bone marrow of a spontaneously diseased osteomalacic rat, he isolated a gram-positive diplococcus with a tendency to form chains in fluid media. With this organism he was able to produce rachitic lesions in young rats.

Joseph Koch<sup>2</sup> in the course of experiments with *Streptococcus longus*, injected intravenously into young dogs, observed after a considerable lapse of time hypertrophy of the bones, particularly at the epiphyseal and costochondral junctions, disproportionately large head and thorax, and marked changes in the teeth. Histologically there was an irregular line of ossification, a region of osteoid cartilage, between the cartilage and bone, and the marrow between the trabeculae of the spongy bone resembled the "gerustmark" of infantile scurvy.

Pappenheimer<sup>3</sup> describes the production of rickets in rats by the injection of a suspension of bone marrow from a rachitic animal.

In addition to the experimental work of Murpurgo, Koch, and Pappenheimer, there are two clinical studies, one by Ausset<sup>4</sup> and another by Coplans,<sup>5</sup> which speak strongly for infection as the important etiologic factor in scurvy and rickets.

Our work was undertaken primarily to determine whether certain small stained bodies seen in sections of scurvy lesions of guinea-pigs were bacteria.

Scurvy was produced in the guinea-pigs by feeding whole milk and oats, hay, and water. The diseased joints, muscles, and lymph glands

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<sup>1</sup> Verhandl. d. deutsch. path. Gesellsch., 1900, 3, p. 40.

<sup>2</sup> Centralbl. f. Bakteriöl., R., 1913, 57, p. 250.

<sup>3</sup> Jour. Exper. Med., 1914, 20, p. 477.

<sup>4</sup> Ann. de méd. et chir. inf., 1904, 8, p. 289.

<sup>5</sup> Lancet, 1914, 1, p. 1714.

were ground up in a sterile chamber. Blood-agar-plate cultures and shake cultures in ascites-dextrose agar were made of this material. After 20 hours' incubation at 37 C. small greenish colonies were present in the plates, and small pinpoint-sized colonies in the shake cultures. (In our first cultures many staphylococcus colonies were obtained, which we now feel sure were contaminations as the development of a more careful technic practically excluded them from the growths.) We isolated organisms with many similar characteristics from the lesions in various parts of the bodies of 9 guinea-pigs having well-developed experimental scurvy. Six of these animals had been fed on milk, and 3 on oats, hay, and water.

The organisms isolated were gram-positive with occasional gram-negative ones intermingled. They occurred in pairs and short chains and occasionally in tetrads and small irregular groups. As a rule they were small spherical cocci but in old cultures there was great irregularity in size and shape. On blood agar they first appeared as small pinpoint-sized greenish colonies with a narrow greenish zone of hemolysis, which in from 48 to 72 hours had a tendency to become clear. The entire diameter of the colonies and hemolytic zone varied from 1.5 to 3 mm. but was usually not more than 2 mm.

Seventeen strains were tested for their acid-production in various sugar media, made up of litmus broth and 1% each of one of 8 sugars, dextrose, lactose, maltose, saccharose, raffinose, mannite, inulin, and salicin. The reactions varied so that definite grouping by this method alone seems out of the question.

Twenty rabbits and 19 guinea-pigs were inoculated in the circulating blood with these organisms. The rabbits were the more susceptible, altho only 1 rabbit died from the effects of the injection. Six were killed and examined and cultures made from some of the lesions and from the heart blood of 4. In every instance organisms microscopically and culturally identical with the ones injected were recovered from the lesions but not from the heart blood. The rabbits all showed signs of muscular stiffness especially in the hind legs. They did not move around to any great extent, and became more and more emaciated. The one rabbit which died lost 240 gm. in weight in the 20 days following the injections.

The characteristic lesions in all the rabbits examined were small hemorrhages in the muscles, especially near the tendons' ends, the muscle bundles being of a mottled yellowish-red appearance and showing marked friability. This latter condition was observed in the animals which had lived over a week or 10 days. Cultures were not made of tissues from animals killed under 10 days. Hemorrhages were found beneath the periosteum in the region of the lower incisor teeth and the acetabulum and ribs. The marrow near the ends of the long bones had definite macroscopic hemorrhagic regions. Since this

work is concerned with experimental scurvy in guinea-pigs, no further mention will be made of the results in rabbits.

Of the guinea-pigs receiving single intracardiac injections, 10 developed definite gross lesions. There were enlargements of the wrists, knees, or both, and cyanosis in the gums. The earliest that any change was noticed was 9 days after injection. Only 1 guinea-pig succumbed to the inoculation.

Four guinea-pigs, 2 on regular diet and 2 on oats, hay, and water, received 7 subcutaneous injections of from 5 to 7 millions of living streptococci every 3 days. The animals all lost in weight. The two on oats, hay, and water died on the 20th and 21st days respectively. These had some lesions resembling scurvy, but mild as compared with those in 2 control guinea-pigs receiving the same diet but no injections. One of the pigs on the ordinary diet died on the 28th day with no gross evidence of scurvy. The other recovered.

Complement-fixation tests were made on the blood of some of the scurvy guinea-pigs, with, as antigen, a streptococcus isolated from the first typical scurvy examined. There was apparently slight binding in the serum of the diseased animals, while the controls were completely negative. The blood of one animal inoculated subcutaneously had very strong complement-fixation properties.

The joints and muscles from 2 guinea-pigs which a month previously had received intraperitoneal injections of sputum treated with sodium hydrate, were cultured and in each instance one colony of streptococci, apparently similar to the one already described, was recovered. Strains of these organisms injected into rabbits and guinea-pigs produced negative results. Muscles from normal guinea-pigs when cultured were negative.

We also made cultures from different parts of the small and large intestines in the diseased animals but failed to find the organisms looked for, probably because of other bacteria present. In the mouths, however, of both healthy and diseased guinea-pigs at least 50% of the bacteria belonged to the green-producing streptococcus group. Previous work by others had brought out this fact. Blood cultures were negative in every instance.

Tissues were examined from 8 guinea-pigs injected intracardially with organisms recovered from the lesions of guinea-pigs suffering from scurvy produced by feeding milk; these included the knee joint from 6, the wrist joint from 6, the lower jaw from 2, and the elbow joint 1. As might be expected in view of the fact that guinea-pigs affected with scurvy produced by abnormal feeding recover when

placed on a normal diet, the alterations in normally fed guinea-pigs injected intracardially with the organisms isolated from lesions of scurvy were much milder than those occurring in the condition produced by feeding, but were of the same general character and were found in the same locations.

Hemorrhages had occurred in the bone marrow, periosteum, and muscles but were less numerous and less extensive as were also the necroses. There was in some cases a very marked increase in the number of eosinophile cells. This was also frequently observed in the milk-fed pigs. Similarly the early changes were hemorrhages and necroses and occasionally there was evidence of thrombosis of the veins in the bone marrow of the long bones. Alterations in the bone marrow and periosteum of the maxillae were more conspicuous than those in the tooth pulp. Apparently these injuries heal rather rapidly and completely, as in guinea-pigs living from 80 to 90 days after inoculation about the only changes to be noted were those in the bone marrow. Indeed it would hardly seem possible with a single inoculation to produce lesions of any greater severity or of long duration with the animal living under normal conditions and therefore those most favorable for recovery.

#### SUMMARY

Bacteriologically, cultures of crushed tissue resulted in the isolation of a diplococcus of low virulence with a tendency to form chains and produce green on blood agar.

Pure strains of these organisms inoculated into the circulation of guinea-pigs and rabbits living under ordinary conditions (a mixed diet consisting of green vegetables, hay, and oats) gave rise in most instances to hemorrhagic and other lesions in the bones, joints, muscles, lymph glands, or gums.

Streptococci of the same type as those injected were recovered from the lesions in these animals as late as 40 days after a single intracardiac or intravenous injection.

Bacteria resembling the organisms described were frequently seen in the microscopical sections of the scurvy lesions.

When animals, which had artificially received these streptococci in the circulation had their resistance kept high by proper feeding, the lesions produced did not have the same tendency to progress that was seen in animals receiving an unbalanced diet.

Cultures of the heart blood from the affected guinea-pigs were sterile and passage of blood from an affected animal to a normal animal failed to produce the disease.

# LESIONS IN RABBITS PRODUCED BY STREPTOCOCCI FROM CHRONIC ALVEOLAR ABSCESES \*

A. M. MOODY

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The literature contains no reference to results from the injection of animals with strains of *Streptococcus viridans* from chronic alveolar abscesses in persons who, in other respects, are in perfect health. This, it seems to me, is a field which has to be thoroughly studied before one has the right to conclude that bacteria isolated from individuals with more remote processes possess an elective affinity for similar tissues in animals into which they may be injected. The idea is one which should be applied to the study of streptococci from other foci of infection as well as the one considered in this paper. Rosenow's<sup>1</sup> valuable work on the elective affinity of streptococci demands a complementary study of freshly isolated strains from normal individuals. His miscellaneous group may include, to a certain degree, the results of such a study. He has demonstrated, moreover, that laboratory strains lose the power to produce the high percentage of lesions produced by strains recently isolated from foci of infection. Henrici,<sup>2</sup> working with Hartzell, made bacteriologic examinations and some animal inoculations with material from chronic alveolar abscesses in patients with systemic disease. He described especially the lesions of the heart, aorta, kidneys, and joints in 24 animals which had been injected with large doses of *Streptococcus viridans*.

Since this paper is concerned primarily with animal experimentation in connection with *Streptococcus viridans* from chronic alveolar abscesses, no mention will be made of the literature on the bacteriology of such foci of infection.

With the reports cited in mind, a study was begun to determine as far as possible what *Streptococcus viridans* isolated from chronic alveolar abscesses would do when injected into the blood of young rabbits. The points to be considered were the following: (1) What is the relation of *Streptococcus viridans* in chronic alveolar abscess to systemic disease? (2) What properties of elective localization does

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<sup>1</sup> Jour. Am. Med. Assn., 1915, 65, p. 1687.

<sup>2</sup> Ibid., 64, p. 1055.

this organism possess? (3) What kind of lesions follow injection of *Streptococcus viridans* isolated from individuals who otherwise are in perfect health? (4) Do the lesions occurring in rabbits injected with this streptococcus warrant the conclusion that similar lesions are apt to follow the more gradual invasion of the human body with a like strain of the organism in question?

For the study of these questions, a definite but simple technic was followed in the collection of material, in the bacteriologic examination, and in the method of injection.

The material examined was collected by Dr. T. L. Gilmer from 55 of his patients. The abscesses selected were all of the chronic walled-off variety, located at the roots of the teeth and having no connection with the mouth cavity. The pus was received in sterile sealed glass capillary pipets, into which it had been drawn free from contamination with mouth bacteria. Of the 55 patients 49 yielded cultures suitable for animal experimentation. Of the remaining six 4 gave sterile material, and 2 gave pus that had to be discarded because of the presence of contaminating organisms.

The pus, even tho extremely small in amount, was diluted in from 1 to 2 c.c. of broth and thoroughly mixed. Cultures were made in 4 different ways, one-fourth of the amount being used for each method, as follows: aerobic cultures in blood-agar plates, and in ascites-dextrose broth, anaerobic cultures on blood-agar slants (Wright's method), and shake cultures in tall columns of ascites-dextrose agar. The ascites-dextrose broth was put up in centrifuge tubes containing just 10 c.c. of the medium.

The aerobic growths yielded in nearly every instance pure cultures of *Streptococcus viridans*. Occasionally stray colonies of staphylococci and hemolytic streptococci were found. In the first 12 cases of this series, only blood-agar plates and occasional anaerobic slants were used.

The bacteriologic technic used was essentially that followed by Rosenow, to whom I am indebted for advice as to details of method. A smaller dosage of organisms for injection was employed in this series than was used by Rosenow, and the animals were allowed to live a longer time after injection.

At the end of from 18 to 20 hours' incubation at 37 C. the broth culture was sedimented at high speed (4000 revolutions per minute), the sediment was taken up in 5 c.c. of physiologic salt solution, and from 1.5 to 2 c.c. of this were immediately injected into the ear vein of each of 2 rabbits. Blood-agar-plate cultures were then made of the suspension to determine the organism and also the approximate number contained in each cubic centimeter. This number averaged about 125 millions to the cubic centimeter. The animals injected with streptococci isolated from the first 12 patients received from two to three times the dose given in the remainder of the series.

At the same time transfers were made from the isolated green colonies of streptococci in the blood-agar plates of the original material. These were inoculated into ascites-dextrose broth to be used after 18 hours' incubation for the injection of 2 more rabbits in the manner described.

The rabbits used averaged about 1000 gm. in weight. After injection they were allowed to live for from 5 to 8 days (in only a few instances longer), and then were killed with chloroform and immediately examined. The routine of the postmortem was systematically to examine every part of the body, including the skin and nervous system. All pathologic conditions were recorded as soon as discovered.



The patients from whom material was received were divided into 3 groups with respect to a study of selective localization: Group A comprised 15 patients suffering from chronic alveolar abscess and articular rheumatism; Group B, 15 patients suffering from chronic alveolar abscess and some systemic disorder other than articular rheumatism; Group C, 19 patients suffering from chronic alveolar abscess unaccompanied by any other illness.

TABLE 1  
THE PERCENTAGE OF RABBITS SHOWING LESIONS FOLLOWING INTRAVENOUS INJECTION OF STREPTOCOCCI FROM CHRONIC ALVEOLAR ABSCESSES IN MEMBERS OF GROUP A

Case	Clinical Diagnosis in Addition to Chronic Alveolar Abscess and Articular Rheumatism	Number of Animals In- jected	Percentage of Animals Showing Lesions in					
			Appen- dix	Stomach		Gall- blad- der	Pan- creas	Intes- tines
				Hemor- rhage	Ulcer			
2	Muscular rheumatism.....	2	0	50	0	0	0	0
4	.....	2	0	50	50	50	50	50
5	Nervousness.....	2	0	50	0	0	50	0
7	.....	2	0	0	0	50	0	0
8	.....	5	0	0	0	0	20	0
11	Neuritis.....	4	0	100	75	25	75	0
12	Endocarditis.....	4	0	50	25	0	0	0
14	Myositis.....	4	0	25	0	25	0	0
15	Myocarditis; nephritis.....	4	0	50	0	0	0	0
26	.....	3	0	33	0	0	0	0
33	Maxillary sinus infection.....	4	25	25	0	0	0	0
36	Muscular rheumatism; eye lesion.	2	0	50	0	0	0	0
44	.....	2	0	0	0	0	0	0
46	Endocarditis.....	4	0	25	0	0	0	0
47	.....	4	0	25	0	0	0	0

TABLE 2  
THE PERCENTAGE OF RABBITS SHOWING LESIONS FOLLOWING INTRAVENOUS INJECTION OF STREPTOCOCCI FROM CHRONIC ALVEOLAR ABSCESSES IN MEMBERS OF GROUP B

Case	Clinical Diagnosis in Addition to Chronic Alveolar Abscess and Systemic Disease	Number of Animals In- jected	Percentage of Animals Showing Lesions in					
			Appen- dix	Stomach		Gall- blad- der	Pan- creas	Intes- tines
				Hemor- rhage	Ulcer			
6	Neuritis, abdomen ?.....	2	0	50	50	0	0	0
9	Appendicitis recently; rheuma- tism 5 years ago.....	4	0	50	25	25	25	25
10	Herpes zoster; acute gastric ulcer	4	0	50	0	0	0	0
16	Gastritis; sciatica.....	3	0	0	0	33	0	0
17	.....	4	0	50	25	0	25	0
23	Myocarditis.....	4	0	50	25	0	0	0
25	Sinus infection.....	4	0	50	0	0	0	0
28	.....	4	0	25	0	0	0	0
29	.....	4	0	50	0	0	0	0
34	Myositis.....	4	0	75	0	0	0	0
35	Chronic duodenal ulcer.....	4	0	25	0	0	0	0
38	.....	4	0	100	0	0	0	0
51	Myositis.....	4	0	100	0	0	0	0
50	Anemia.....	4	25	0	0	0	0	25
55	Acute gastric ulcer.....	4	0	75	75	0	0	0

Lesions were produced more frequently in the stomach, muscles, joints, endocardium, kidneys, and jaws in the order mentioned. Strains isolated from the patients having articular rheumatism (Group A), as a rule, produced a higher percentage of lesions in the usual regions of localization than did strains from patients in the other groups. There were, however, certain variations (see charts), the most notable being the production of hemorrhages in the stomach by organisms from

TABLE 1—*Continued*

THE PERCENTAGE OF RABBITS SHOWING LESIONS FOLLOWING INTRAVENOUS INJECTION OF STREPTOCOCCI FROM CHRONIC ALVEOLAR ABSCESSSES IN MEMBERS OF GROUP A

Percentage of Animals Showing Lesions in													
Joints	Peri- car- dium	Endo- car- dium	Myo- car- dium	Mus- cles	Kid- ney	Lungs	Skin	Eye	Jaw	Thy- mus	Thy- roid	Aor- ta	Sci- atic
0	0	0	0	50	0	0	0	0	50	0	0	0	0
50	0	0	0	100	0	0	0	0	0	0	0	0	0
50	0	50	0	50	0	0	0	0	50	0	0	0	0
0	0	0	0	50	0	0	0	0	0	0	0	0	0
40	0	0	0	20	0	0	0	0	20	0	0	0	0
50	0	75	0	75	25	0	0	0	25	0	25	0	0
75	0	50	0	75	25	0	0	0	0	0	25	0	0
75	0	50	0	50	75	0	0	0	0	0	0	0	0
50	0	0	50	100	50	0	0	0	25	0	0	0	0
68	0	0	33	100	0	66	0	0	0	0	0	0	0
0	0	0	25	25	0	0	0	0	25	25	0	25	0
0	0	0	0	0	0	0	0	0	0	0	0	0	25
0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0	0	0	0
25	0	25	0	0	0	0	0	0	0	0	0	0	0

TABLE 2—*Continued*

THE PERCENTAGE OF RABBITS SHOWING LESIONS FOLLOWING INTRAVENOUS INJECTION OF STREPTOCOCCI FROM CHRONIC ALVEOLAR ABSCESSSES IN MEMBERS OF GROUP B

Percentage of Animals Showing Lesions in													
Joints	Peri- car- dium	Endo- car- dium	Myo- car- dium	Mus- cles	Kid- ney	Lungs	Skin	Eye	Jaw	Thy- mus	Thy- roid	Peri- to- neum	Blad- der
0	0	0	0	0	0	50	0	0	50	0	0	0	0
75	0	75	0	75	50	50	0	25	25	0	0	0	0
50	25	0	0	100	25	0	25	0	0	25	25	0	0
33	0	33	0	33	0	0	0	0	0	0	0	33	0
0	0	50	0	0	0	0	0	0	75	0	0	0	0
25	25	25	25	0	25	25	0	0	25	0	0	0	0
25	0	0	0	25	0	25	0	0	0	0	0	0	0
25	0	0	0	25	0	0	0	0	0	25	0	0	25
0	0	0	0	50	0	0	0	0	25	25	0	25	0
0	0	0	0	0	25	0	0	0	0	0	0	0	0
25	0	25	0	0	0	0	0	0	0	0	0	0	0
50	0	100	0	25	25	0	0	0	0	0	0	0	0
0	0	0	0	50	0	0	0	0	0	0	0	0	0
25	0	25	25	75	0	0	0	25	0	0	0	0	0
0	0	0	0	0	0	0	0	0	50	0	0	0	0

Groups B and C. The gross lesions were chiefly hemorrhages of varying degrees of intensity and, in many instances, suppuration.

In the stomach mucosa, in a high percentage of the animals injected, there were small hemorrhages without any sign of ulceration. When present, the ulceration was usually a hemorrhagic erosion rather than a true ulceration. The true ulceration into the deeper tissues with poly-

TABLE 3  
THE PERCENTAGE OF RABBITS SHOWING LESIONS FOLLOWING INTRAVENOUS INJECTION OF STREPTOCOCCI FROM CHRONIC ALVEOLAR ABSCESSSES IN MEMBERS OF GROUP C

Case	Clinical Diagnosis in Addition to Chronic Alveolar Abscess	Number of Animals Injected	Percentage of Animals Showing Lesions in					
			Appendix	Stomach		Gall-bladder	Pancreas	Intestines
				Hemorrhage	Ulcer			
1		2	0	50	0	0	0	0
3	Blood pressure, 240.....	2	0	0	0	0	0	0
13		3	0	33	0	0	0	0
18		4	1	75	50	0	25	0
19	Fistula.....	4	0	50	0	0	0	0
20	Appendicitis 2 years ago.....	4	0	75	25	25	0	0
21		4	0	50	0	25	0	25
22		4	0	75	0	0	0	0
24		4	0	50	25	25	25	0
27		4	0	25	0	0	25	0
30	Recently acute.....	4	0	0	0	0	0	0
31	Slight anemia.....	4	0	25	0	0	0	0
32		4	0	25	0	0	0	0
40		4	0	25	25	0	0	0
43		4	0	50	0	0	0	0
45		4	0	25	0	0	0	0
48		4	0	50	0	0	0	0
53		4	0	0	0	0	0	0
54		4	0	25	25	0	0	0

TABLE 4  
COMPARISON OF GROUPS A, B, AND C WITH RESPECT TO THE AVERAGES OF THE PERCENTAGES GIVEN IN TABLES 1, 2, AND 3

Group	Number of Cases	Number of Animals Injected	Percentage of Animals Showing Lesions in						
			Appendix	Stomach		Gall-bladder	Pan-crease	Intes-tines	Joints
				Hemor-rhage	Ulcer				
A	15	50	1.7	35.7	10.0	10.0	13.0	3.3	35.4
B	15	57	1.7	50.0	13.3	7.2	3.3	3.3	22.2
C	19	71	0.0	37.3	7.9	3.9	3.9	1.3	21.0
Total	49	178	1.1	41.0	10.4	7.0	6.7	2.6	26.2

morphonuclear infiltration and inflammatory exudation was found in but few instances.

The lesions in the muscles were, to the naked eye, regions of hemorrhage, and were usually located near the tendinous end, tho frequently occurring also in the belly of the muscle.

Suppuration was most common in the joints. Of 47 rabbits having joint lesions, only 3 showed hemorrhage alone as the gross pathologic lesion. The remaining 44 rabbits had macroscopic pus in one or more joints.

In the heart small hemorrhages occurred in the endocardium, especially in the right ventricle near the base of the tricuspid valve.

TABLE 3—*Continued*

THE PERCENTAGE OF RABBITS SHOWING LESIONS FOLLOWING INTRAVENOUS INJECTION OF STREPTOCOCCI FROM CHRONIC ALVEOLAR ABSCESSES IN MEMBERS OF GROUP C

Percentage of Animals Showing Lesions in												
Joints	Peri- car- dium	Endo- car- dium	Myo- car- dium	Mus- cles	Kid- ney	Lungs	Skin	Eye	Jaw	Thy- mus	Ad- renal Blad- der	Sci- atic
0	0	0	0	50	0	0	0	0	50	0	0	0
0	0	50	0	50	100	0	0	0	0	0	0	0
0	0	33	0	33	33	33	0	33	66	0	0	0
25	0	0	0	25	0	25	0	0	50	0	25	0
75	0	0	0	75	50	0	0	0	0	0	0	0
50	0	75	0	25	0	0	0	0	0	0	0	0
25	0	0	25	50	75	0	0	0	25	0	0	0
25	0	0	0	25	25	25	0	0	0	0	0	0
0	0	25	25	25	0	0	0	0	0	0	0	0
0	0	25	0	0	0	0	0	0	0	0	0	0
50	0	0	0	50	0	0	0	0	25	0	0	0
25	0	25	75	75	75	0	0	0	0	0	0	0
0	0	0	0	0	50	0	0	0	0	0	0	0
25	0	50	0	25	0	50	0	0	25	0	0	0
25	0	25	0	25	25	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	25	25	0	0
25	0	75	0	75	0	0	0	0	75	0	0	25
25	0	25	0	0	0	0	25	0	0	0	0	0
0	0	0	25	75	25	50	0	0	50	0	0	25

TABLE 4—*Continued*

COMPARISON OF GROUPS A, B, AND C WITH RESPECT TO THE AVERAGES OF THE PERCENTAGES GIVEN IN TABLES 1, 2, AND 3

Percentage of Animals Showing Lesions in									
Pericar- dium	Endocar- dium	Myocar- dium	Mus- cles	Kid- ney	Lungs	Skin	Eye	Jaw	Thy- mus
0.0	16.7	7.2	46.3	13.3	6.0	0.0	0.0	13.0	1.7
3.3	22.2	5.0	30.5	10.0	10.0	1.7	3.3	16.7	5.0
0.0	22.7	7.9	35.9	24.1	9.6	1.3	1.7	20.3	1.3
1.1	20.5	6.7	37.6	15.8	8.5	1.0	1.7	16.7	2.7

These hemorrhages were found in all parts of the heart. In only one instance (Rabbit 488 inoculated with material from Case 3) was there a well-developed vegetative mitral endocarditis. This animal, which was killed 8 days after injection, had, in addition to the heart lesion, multiple infarcts in the kidneys and spleen.

The kidneys revealed small hemorrhagic foci usually beneath the capsule and scattered throughout the cortex. There were very few instances of the development of an acute diffuse nephritis, but many instances of marked acute fatty changes.

In the lower jaw below the incisor teeth and beneath the periosteum, there frequently were seen marked hemorrhages similar to those

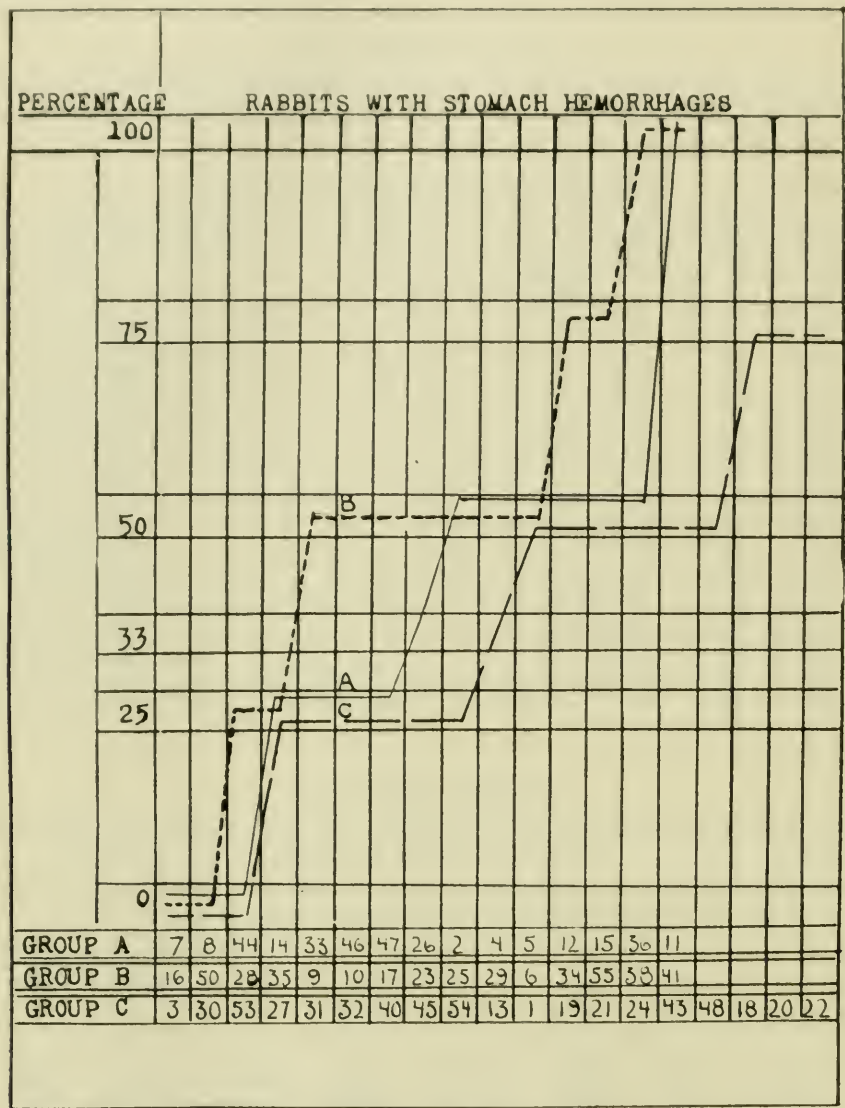


Chart 2. Comparative study of stomach hemorrhages occurring in rabbits injected with streptococci from the various members of Groups A, B, and C.

described by Jackson and Moody.<sup>3</sup> These hemorrhages were not of an inflammatory nature, tho bacteria could be demonstrated in them.

A suppurative cholecystitis developed in one animal (Rabbit 552) injected with streptococci from Case 14. This rabbit was allowed to

<sup>3</sup> Jour. Infect. Dis., 1916, 19, p. 511.

live 13 days after injection. *Streptococcus viridans* was recovered from the pus in pure culture. The other instances of gallbladder involvement were characterized by the presence of small hemorrhages in the mucosa.

Lesions in the other organs and tissues of the inoculated animals were marked by the presence of small focal hemorrhages.

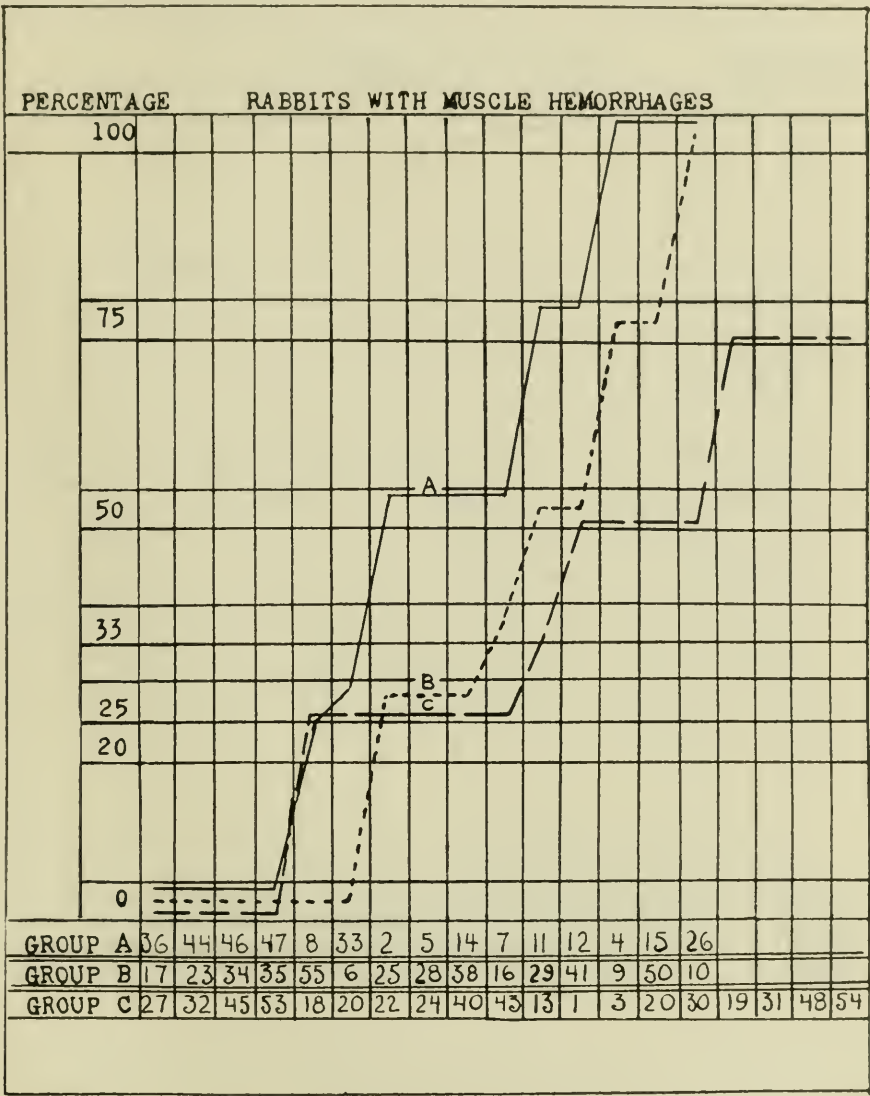


Chart 3. Comparative study of muscle lesions occurring in rabbits injected with streptococci from the various members of Groups A, B, and C.

Microscopical examination, as far as made, corroborated the gross findings. Cultures of the lesions and pus were made only in selected instances, as all animals received relatively large doses of living organisms which, if recovered from the tissues, would not have given any new information.

SUMMARY AND CONCLUSIONS

*Streptococcus viridans* may cause disease in various parts of the rabbit's body.

The lesions produced by organisms from persons without systemic disease are identical in character with those produced by organisms

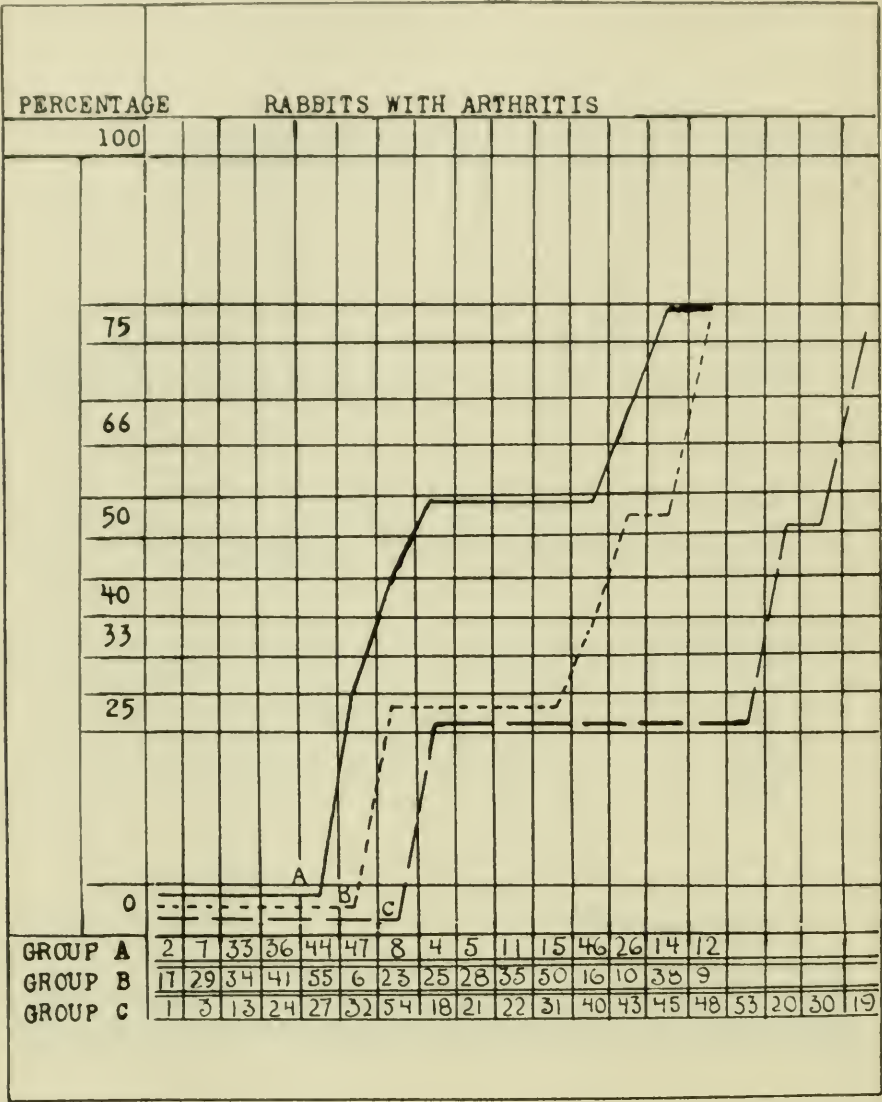


Chart 4. Comparative study of joint lesions occurring in rabbits injected with streptococci from the various members of Groups A, B, and C.

from patients with systemic infections. The fact that the former do not produce lesions quite as frequently as the latter indicates a difference between the organisms only in degree of virulence.

Whether the lesions produced in rabbits by streptococci from persons who, aside from their alveolar abscesses, are in perfect health,

warrant the conclusion that these persons would develop similar conditions were the foci of infection not removed, is a question which cannot be satisfactorily answered. Many of them, at any rate, are well past middle age and otherwise in good health.

There is here abundant evidence for and against the property of elective affinity on the part of streptococci. Therefore, one must conclude that the localization of *Streptococcus viridans* cannot be determined wholly by some property inherent in the organism itself.



# THE ETIOLOGY OF CHOLECYSTITIS AND GALL-STONES AND THEIR PRODUCTION BY THE INTRAVENOUS INJECTION OF BACTERIA \*

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The lodgment and growth of bacteria in the gallbladder have been considered chiefly as accidental, notwithstanding the fact that bacteremia occurs in numerous diseases in which cholecystitis is absent. Attempts by investigators to produce cholecystitis by simple intravenous injection of various bacteria have usually resulted in failure. The typhoid bacillus, however, quite irrespective of its source, has given positive results in the hands of Blachstein,<sup>1</sup> Welch,<sup>2</sup> Cushing,<sup>3</sup> Koch,<sup>4</sup> Gay and Claypole,<sup>5</sup> and Nichols.<sup>6</sup>

During the course of experiments<sup>7</sup> with laboratory strains of streptococci, I noted that lesions of the gallbladder followed intravenous injection of these strains only when the virulence had attained a certain point. Two years ago I reported a case<sup>8</sup> in which cholecystitis had occurred 10 days after tonsillitis, and in which a streptococcus had been isolated from the wall of the gallbladder, and from the centers of the newly formed gallstones. This streptococcus had shown a far greater affinity for the gallbladder in animals than had streptococci from other diseases.<sup>9</sup>

In this paper I wish to record the results of cultures by special methods from the liquid contents of the gallbladder, from the centers of gallstones, from the wall of the gallbladder, and from the adjoining lymph glands removed at operation, in a series of cases of cholecystitis, and the results of animal experiments with the bacteria thus isolated. These results, it is believed, throw definite light on the mechanism of the occurrence of cholecystitis and gallstones.

## TECHNIC

The gallbladder or adjacent lymph glands were removed aseptically with as little exposure to the air and skin as possible, covered at once with sterile gauze, and taken unopened to the laboratory. The contents were collected in pipets; a portion of the gallbladder (0.5 cm. by 1 cm.) was excised, and one or more stones, if present, were saved for cultures. The pieces of tissue from

\* Received for publication May 3, 1916.

the gallbladder and the lymph glands were washed twice in salt solution, the surfaces flamed or seared with a blade, and then the tissue emulsified in a mortar in a specially devised sterile air chamber.<sup>10</sup>

Cultures from the gallstones were made from pulverized material taken from the centers by means of a small dental burr, after the surfaces had been seared with a blade. Control cultures from more superficial portions were made in some instances. The cultures were made in tall columns of liquefied ascites (10%) dextrose (1%) agar, in ascites dextrose broth, in litmus milk, and on blood-agar slants. In some instances pieces of sterile tissue, guinea-pig heart or kidney, were added to the bottom of the shake cultures in agar. The cultures were incubated at from 35 to 37 C. for at least 10 days before being discarded, and were examined daily.

The bacteria for injection were usually from single colonies in the shake cultures, and had been grown in tall columns (12 cm.) of ascites dextrose broth containing sterile tissue, for from 18 to 28 hours, centrifugated, the clear broth poured off, and the bacteria suspended in salt solution so that 1 c.c. contained the growth from 15 c.c. of the broth culture. The injections were made intravenously through a 23-gauge needle with a glass syringe, sterilized by boiling. The portion of the suspension left over was preserved in the ice chest for further injections in case the results warranted further study. This method was found to preserve the elective property of the bacteria in the presence of pieces of sterile tissue for a longer time than repeated subcultures. In one instance it endured for as long as 20 months (Case 140). Blood-agar-plate cultures and smears were made from the suspensions at the time of the injections to prove the viability of the organisms at the time of injection and to be used in the study of cultural features. In most experiments the injections were made some hours after the feeding period. The dogs were fed meat and bread or dog biscuits, the rabbits and guinea-pigs liberal amounts of greens, bread, or oats and hay.

The autopsies were made as soon after death as possible. A careful inspection in a bright light aided with a hand lens was made for focal lesions. Cultures were made routinely from blood, bile, joint fluid, and the emulsified tissues of the wall of the gallbladder. The pieces of tissue saved for microscopical study were fixed in Kaiserling's solution, Zenker's solution, 10% formalin, or in 80% alcohol.

#### RESULTS OF THE CULTURES IN HUMAN CHOLECYSTITIS

Of the patients in whom sex was noted, 25 were females and 13 males. The youngest was 18 years of age, the oldest 68. There were 28 between the ages of 30 and 60 years. The duration of symptoms in the different cases ranged between 6 weeks and 25 years.

The cultures were made from material removed at operation in 47 cases. The bile or other fluid content of the gallbladder (Table 1) was cultured in 29 cases, 13 of which showed no bacteria. In the remaining 16 cases the streptococcus was not found in pure culture, being found in conjunction with the colon bacillus in 5 instances, and with other bacteria in 2. The colon bacillus was found in pure culture 4 times.

Cultures from the nuclei of the common types of gallstones were made in 33 cases, showing no bacteria. In the remaining 29 cases the

streptococcus was isolated in pure culture in 17, and the colon bacillus in pure growth in 1. The streptococcus occurred with the colon bacillus in 3 cases, and with other bacteria in 4. Cultures made from 4 typical cholesterol stones (not given in tables) showed no streptococci; 2 were sterile, 1 yielded a few colonies of a diphtheroid bacillus, and 1 the colon bacillus.

Cultures from the wall of the gallbladder were made in 32 cases, 5 of which were sterile. Of the remaining 27 the streptococcus was found in pure culture in 10, the colon bacillus in 1, mixtures of streptococcus and colon bacillus in 8, and the streptococcus with other bacteria in 3.

Of the cases in which the fluid contents were sterile the streptococcus was isolated from the wall of the gallbladder in 8, and from the center of gallstones in 6.

The adjacent lymph glands were cultured in 8 cases; 1 gave no bacteria, 4 showed pure cultures of the streptococcus, 1 the streptococcus and a diphtheroid bacillus, and 1 a pure culture of colon bacillus.

*Bacillus welchii* was found in conjunction with other bacteria in the gallstones in 7 cases, and in the wall of the gallbladder and in the adjacent lymph glands in 1 instance each. It was not obtained in cultures from the fluid contents. *Staphylococcus aureus* alone was present in the contents and wall in 1 case, and in mixture with the colon bacillus in 1 case of acute cholecystitis. Diphtheroid bacilli were found pure in the gallbladder wall in 1 case, and in mixture with streptococci in the contents in 1, in the wall in 2, and in a lymph gland in 1. Fusiform bacilli were found in conjunction with the colon bacillus or the streptococcus in the contents and in the wall in 2 cases. *Bacillus mucosus* was found in conjunction with *Staphylococcus aureus* in the contents in 1 case, and in conjunction with the colon bacillus and the fusiform bacillus in the wall of the gallbladder in 1 case, both in acute cholecystitis. Unidentified strictly aerobic bacilli and cocci, which were found occasionally, were considered contaminations. The typhoid bacillus was isolated from the center of a gallstone in 1 case.

The cultures from the outer portion of the gallstones were sterile when the fluid contents were sterile, and contained a bacterial flora similar to that found in the fluid contents when the latter were infected. The nuclei of the stones, on the other hand, often yielded pure cultures of streptococci, irrespective of the bacterial flora of the fluid contents. In 12 cases the streptococcus was isolated both from the center of the stone and from the wall of the gallbladder. In 5 cases of advanced

chronic cholecystitis without stones, streptococci were isolated from the wall of the gallbladder. In 5 cases of chronic catarrhal cholecystitis without stones, in which the changes were slight, streptococci were isolated in 1, a few colon bacilli in 2, and no bacteria in 2. In 5 cases of chronic catarrhal cholecystitis with stones, the streptococcus was isolated from the center of the stones in 2 cases, and a diphtheroid bacillus in 1. In 9 cases of acute cholecystitis in the first attack or during an acute exacerbation of a chronic cholecystitis, cultures showed the streptococcus alone or in combination with other bacteria in the wall in 7 cases, and in the stones in 5; the colon bacillus was found in 4 of these, *Bacillus mucosus* in 2, and the staphylococcus in 2. Streptococci were found in small numbers either in the stone or in the wall of the gallbladder in 4 cases in which the mucous membrane of the gallbladder presented the so-called strawberry appearance.<sup>11</sup>

The gross and microscopic changes in the gallbladders studied were of the usual character. Those in which there were chronic changes, and which had been removed during the quiescent interval, showed fibrosis with little cellular infiltration, while those in which there had been a recent acute attack had hemorrhages and marked infiltration, chiefly in the submucosa and subperitoneal coat. Cross sections at the apex of the gallbladder revealed no noteworthy changes in the nerve trunks. The eosinophilic infiltration of the fibrous tissue was often a striking picture. The bacteria were found in the areas of hemorrhage and infiltration in relatively large numbers, but were also demonstrated in the more or less dense fibrous tissue in the gallbladders with chronic changes only.

#### REVIEW OF IMPORTANT CASES AND ANIMAL EXPERIMENTS

##### CASE 61

A woman, 59 years of age, who had suffered for 15 years from symptoms referable to the gallbladder or the stomach, was operated on during an exacerbation of symptoms August 11, 1914. The gallbladder, full of stones, and a gland the size of a navy-bean at the juncture of the cystic and common ducts, were removed. A rather large indurated ulcer also was found at the lesser curvature of the stomach. Cultures were made from a portion of the gallbladder wall, 0.5 cm. square, showing what appeared to be an infarcted area, and from the bile, the lymph gland, and the centers of two of the stones.

August 12.—Tall tubes of ascites-dextrose and dextrose agar inoculated with the emulsion of the wall yielded altogether 28 colonies of streptococci, while those inoculated with the emulsion of the gland gave 5. All the colonies were in the upper three-fourths of the tubes. In one tube inoculated with a large amount of the emulsion of the gallbladder wall, there were 2 colonies of *B. welchii*. The centers of the gallstones yielded a short-chained streptococcus in dextrose broth. The cultures from the bile remained sterile. Subcultures were

made on blood-agar plates from 6 of the colonies from the wall of the gallbladder, from 2 from the gland, and from the growth in the broth inoculated with material from the centers of the stones. The rest of the colonies were not disturbed.

Aug. 13.—Blood-agar plates showed small grayish-green moist, but discrete, quite opaque colonies of short-chained streptococci in pure culture.

Aug. 25.—Subcultures from original colonies, which had grown to be 2 mm. in diameter, were made, by means of small pipets, into the usual flasks of ascites dextrose broth containing sterile tissue.

Aug. 26.—The broth showed a dense diffuse turbidity with slight sediment and marked acidity. After centrifugation, the supernatant broth was poured off, and the sediment suspended in NaCl solution to be injected into animals.

Sections through the infarcted area near the fundus showed leukocytic infiltration, and easily findable streptococci and *B. welchii* (Fig. 1). Cross sections at the apex of the gallbladder, near the juncture of the cystic duct, showed fibrosis of the interstitial tissue, thickening of the walls of the blood vessel, with moderate leukocytic and round-cell infiltration around blood vessels in the interstitial tissue and submucosa. A few diplococci were found in the areas of leukocytic infiltration. In the interstitial tissue leukocytes were chiefly eosinophils. Nerve trunks and the mucous membrane were quite free from changes.

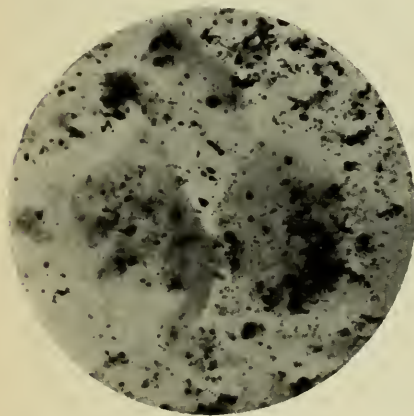


Fig. 2. Photograph of the material found in the hemorrhagic and infiltrated gallbladder of a dog (D 158) after repeated intravenous injections of streptococci. Black gallstones are embedded in the flakes of mucopurulent material. Natural size.

the streptococcus isolated from the gland.

Aug. 28.—It seemed well. Killed. Gallbladder distended with thick greenish-black bile, in which were a large number of black particles from 0.5 to 1.5 mm.

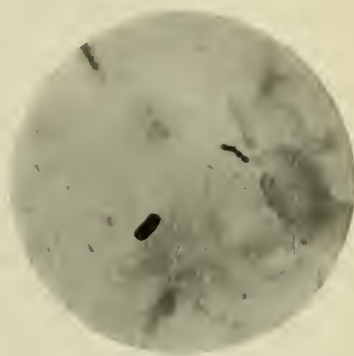


Fig. 1. Chain of streptococci and *Bacillus welchii*, causing infiltration in the gallbladder, Case 61. Gram-Weigert.  $\times 1200$ .

Intravenous injection into 1 dog and 1 rabbit of the streptococcus from the gland, produced localized hemorrhages and edema in the gallbladders of both, while the streptococcus from the wall of the gallbladder as isolated, produced cholecystitis in 2 dogs injected, as well as in 2 dogs after 1 animal passage. Two of 3 rabbits developed no lesions in the gallbladder; the other had cholecystitis. In 1 rabbit and 2 of the dogs with cholecystitis were numerous small black gallstones imbedded in a thick black mucus containing bile (Fig. 2). The opening in the ampulla of Vater in the rabbit was plugged with a similar material. This is interesting because the gallbladder from which the strain was isolated was filled with stones proved to contain streptococci.

*Rabbit 794.*—Injected intravenously, August 26, with the growth from 45 c.c. of an ascites-dextrose-tissue-broth culture of

in diameter. Ducts dilated; bile not to be expressed until the plug of mucus containing soft stones was removed from the ampulla. Wall of the gallbladder thickened and edematous. Few small hemorrhages at the base of the tricuspid valve and in the lung. Joint fluid slightly turbid.

Aug. 29.—Liver, blood, and bile cultures sterile.

*Dog 105.*—Injected intravenously, August 26, with the growth from 90 c.c. of an ascites-dextrose-tissue-broth culture of the streptococcus from the wall of the gallbladder.

Aug. 29.—The animal seemed well. Chloroformed. No noteworthy lesions anywhere except a marked hemorrhagic cholecystitis. Gallbladder wall edematous and hemorrhagic, particularly at the apex; from 0.3 to 0.6 cm. in thickness. Mucous membrane edematous, in places ulcerated and separated from the serous coat by marked hemorrhage; covered with thick adherent bile-stained mucus. A number of small hemorrhages along the cystic duct, but none along the common and hepatic ducts. Joint fluid clear.

Aug. 31.—Cultures in blood agar from the bile, blood, liver, and joint fluid were sterile, while those from the wall yielded a pure growth of streptococci in ascites dextrose agar and broth, and on blood-agar plates. Sections of the gallbladder showed marked hemorrhage in the subperitoneal layer and moderate leukocytic infiltration. This was most marked in the submucosa and in the peritoneal coat, where a moderate number of gram-positive diplococci were found.

#### CASE 85

A man, 60 years of age, who had not been ill until after a misstep from a ladder 5 months previous to examination. Since then he had had pain in the epigastrium, dizziness, and for a time chills and fever. At operation on August 15, 1914, there were disclosed a distended and somewhat thickened gallbladder, marked pancreatitis of the head of the pancreas, and enlarged lymph glands along the cystic and common ducts. The gallbladder contained a large amount of dark bile and masses of tenacious mucus, in which were embedded a very large number of black particles, biliary sand, varying in size from a pin point to a grain of wheat. In smears from the bile and from the centers of the stones was what appeared to be *B. fusiformis*. In cultures from the bile, from the centers of 2 of the stones, and from the wall of the gallbladder there were streptococci and colon bacilli. The organism resembling *B. fusiformis* which was found in smears failed to grow. Two dogs and 1 rabbit injected intravenously with the streptococcus soon after isolation, developed cholecystitis. In the 2 dogs in which injections were made into the portal vein, there were no lesions of the gallbladder or of other organs. The 2 dogs in which the general circulation had been injected, had mild arthritis in addition to the cholecystitis, and 1 had hemorrhage of the stomach.

*Dog 107.*—Injected, August 26, in the leg vein with the growth from 60 c.c. of an ascites-dextrose-tissue-broth culture of the streptococcus from the gallbladder wall in the shake cultures in ascites dextrose agar.

Aug. 28.—Seemed well. Chloroformed. Three circumscribed hemorrhages in the mucous membrane of the gallbladder, and 1 in the mucous membrane of the lesser curvature of the stomach.

Aug. 29.—Cultures from blood, joint fluid, and bile were sterile on blood agar, but those from the blood in dextrose broth gave short-chained streptococci.

*Dog 113.*—On August 26, a small radicle of the portal vein was injected with the growth from 60 c.c. of an ascites-dextrose-broth culture of the streptococcus as isolated from the gallbladder wall.

Aug. 29.—Seemed well. Chloroformed. A localized peritonitis and plastic adhesions at the point of injection. In the gallbladder and elsewhere, no changes.

Aug. 31.—Cultures on blood agar of joint fluid, blood, liver, and bile were sterile, while the broth culture of the bile yielded a pure growth of a short-chained streptococcus.

#### CASE 120

A man, 55 years of age, suffering with chronic cholecystitis and chronic duodenal ulcer was operated on August 20, 1914. There were found a gallbladder with marked sclerosis and thickening, a large indurated ulcer of the duodenum just outside the pylorus, adherent to the head of the pancreas, and a pea-sized lymph gland draining the ulcer. The gallbladder, containing bile and one stone 2 cm. in diameter, and the gland were removed and cultured.

Cross sections of the apex of the gallbladder revealed marked fibrosis of the submucous muscularis and peritoneal coat, thickening of the blood vessels, hemorrhagic infiltration chiefly of the peritoneum and subperitoneum, atrophy of the mucous membrane, and a few diplococci in the hemorrhagic areas.

Cultures from the bile were negative. In the center of the stone were streptococci, colon bacilli, and *B. welchii*. The gallbladder wall and the lymph gland draining the ulcer yielded pure cultures of the streptococcus. These strains were similar in appearance, but those from the lymph gland produced long chains and colonies with a distinct green zone on blood-agar plates, while those from the ulcer produced grayish colonies with no tinge of green.

The streptococcus from the gallbladder wall produced cholecystitis in 2 dogs injected, but no other noteworthy lesions, while the streptococcus from the lymph gland draining the ulcer produced hemorrhage and ulcer of the stomach in 1 of 2 dogs, and in both of 2 rabbits. The dog without lesions in the stomach or duodenum showed no other lesions. One rabbit, in addition to ulcer, had mild arthritis and a few hemorrhages in the subcutaneous tissue and muscles.

*Dog 114.*—Injected intravenously, August 28, with the growth from 30 c.c. of an ascites-dextrose-broth culture of the streptococcus isolated from the wall of the gallbladder.

Aug. 31.—Seemed well. Chloroformed. A number of small hemorrhages from 0.3 to 0.8 cm. in diameter and edema of the gallbladder, especially along its attachment to the liver. No changes in bile ducts, appendix, stomach, duodenum, or pancreas.

Sept. 1.—Blood-agar-plate cultures from the bile gave 6 colonies of streptococci, and cultures from the gallbladder wall yielded 3 colonies of streptococci and 4 colonies of colon bacilli. Blood and joint fluid sterile.

*Dog 109.*—Injected intravenously, August 26, with the growth from 120 c.c. of an ascites-dextrose-broth culture of the streptococcus isolated from the gland draining the ulcer.

Aug. 28.—Seemed well. Chloroformed. No gross lesions except numerous small punctate hemorrhages scattered over 3 circumscribed areas in the pyloric ring. In the centers of 2 of these the mucous membrane was necrotic and ulcerated.

*Rabbit 700.*—Injected intravenously, August 26, with the growth from 15 c.c. of an ascites-dextrose-broth culture of the streptococcus isolated from the gland draining the ulcer.

Aug. 28.—Killed. No gross lesions except ulcer, 0.2 cm. by 0.4 cm., at the juncture of the middle and lower thirds of the stomach, near the lesser curvature. Base hemorrhagic; mucous membrane ulcerated and undermined.

Aug. 29.—Cultures from blood, joint fluid, and bile were negative, while the culture from the ulcer yielded approximately 100 colonies of streptococci.

#### CASE 135

A woman, 68 years of age, with chronic cholecystitis and an acute exacerbation. The attack of cholecystitis with chills and fever had begun 10 days previously. Symptoms of stones in the gallbladder dated back 15 years. The patient had never been jaundiced. At operation, August 22, 1914, there were disclosed a greatly thickened gallbladder containing many stones and mucopurulent material free from bile, an inflamed and dilated common duct, the size of the small intestine, containing 1 large stone, and enlarged lymph glands along the cystic and common ducts. The gallbladder and 1 lymph gland were removed and cultures made.

Aug. 23.—In the cultures from the centers of two of the stones, from the wall of the gallbladder, and from the mucopurulent material, were streptococci and colon bacilli, while in those from the gland there were streptococci only. There were more colonies of colon bacilli than of streptococci from the mucopus and from the centers of the stones, while the wall of the gallbladder showed a preponderance of streptococci.

Cross section of the apex of the gallbladder showed fibrosis, marked dilation of blood vessels, hemorrhage and marked leukocytic infiltration, especially in the submucosa, and numerous bacilli and diplococci both in the mucous membrane and in the hemorrhagic peritoneal coat.

One dog injected intravenously with a mixture of the streptococcus and the colon bacillus as isolated from the stone, developed a marked cholecystitis. Three dogs and 1 rabbit injected with the streptococcus from the stone, all showed cholecystitis. One dog and 1 rabbit in which a small branch of the portal vein had been injected, disclosed no lesions in the gallbladder. One dog in which the gallbladder had been injected directly, developed only a localized cholecystitis and peritonitis around the place of puncture. The streptococci appeared to have been washed out by the bile without having produced lesions of the mucous membranes of the gallbladder or of the bile ducts.

*Dog 102.*—Injected intravenously, August 24, with the growth from 150 c.c. of an ascites-dextrose-broth culture of a mixture of streptococci and colon bacilli from the center of the gallstone.

Aug. 26.—Seemed well. Chloroformed. Gallbladder wall hemorrhagic in areas and extremely edematous; from 0.4 to 0.8 mm. in thickness. Pressure caused marked oozing of a blood- and bile-tinged fluid from the cut surface. Mucous membrane covered with an adherent mucus and necrotic material, but not ulcerated. No hemorrhages in the liver; marked hemorrhage in the first two centimeters of the common duct, and many small hemorrhages in the head of the pancreas. Lymph glands along the ducts enlarged and hemorrhagic on the cut surface. No other focal lesions except hyperemia of the mucous membrane of the small intestine and slight turbidity of the joint fluid.

Aug. 27.—Cultures from the joint fluid negative. The blood and the bile in blood agar yielded only colon bacilli; in broth, both colon bacilli and streptococci. The gallbladder wall and lymph gland gave colon bacilli and streptococci.

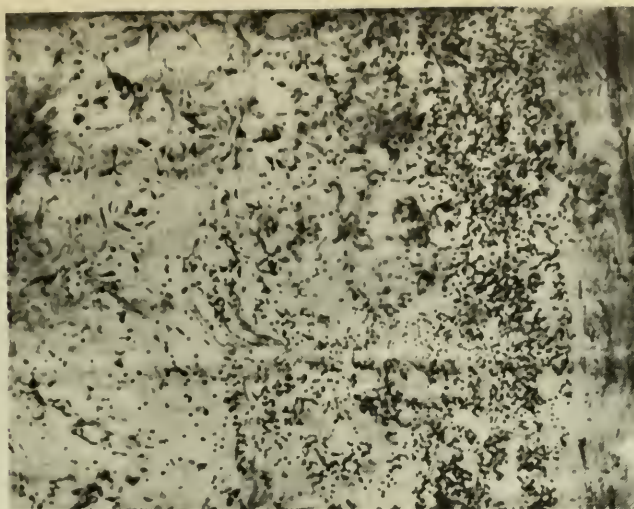


Fig. 3. Section of a gallbladder, Case 140, showing marked fibrosis, marked subperitoneal hemorrhage, and leukocytic infiltration. Hematoxylin-eosin.  $\times 70$ .

*Dog 108.*—Injected intravenously, August 26, with the growth from 90 c.c. of an ascites-dextrose-tissue-broth culture of a pure growth of the streptococcus from the gallstone.

Aug. 29.—Seemed ill. Chloroformed. The gallbladder contained many circumscribed hemorrhages surrounded by moderate edema. These were most marked over the fundus, and along the attachment of the gallbladder to the liver. Mucous membrane hyperemic with a few small hemorrhages. Liver, ducts, and pancreas appeared normal. Joint fluid distinctly turbid.

Aug. 31.—Cultures from the bile and the liver gave a pure growth of the streptococcus, while the blood and the joint fluid remained sterile.

#### CASE 140

A woman, 34 years of age, with acute and chronic cholecystitis. The symptoms suggesting gallstones and cholecystitis had been present for 5 years, but the last attack, during which she had intense pain in the epigastrium, accompanied by a moderate temperature and the development of a leukocytosis, had continued for 20 days. The patient was operated on August 22, 1914, as the symptoms were subsiding. There was an adherent edematous hemorrhagic and much thickened gallbladder, containing 6 stones and much pus. One of the stones was impacted in the cystic duct. The appendix was thin and fibrous, its lumen obliterated. The gallbladder was removed and cultures made from a portion of the wall 1 cm. square from near the fundus, from the center of one stone, and from the fluid contents.

Aug. 23.—The shake cultures in ascites dextrose agar from the center of one of the stones showed approximately 7,200 colonies of the typical streptococcus; those from the emulsion of the wall of the gallbladder yielded 40 colonies of the same streptococcus (Fig. 8); the pus was sterile.

Aug. 24.—Blood-agar-plate cultures from single colonies in the shake culture showed a pure growth of small grayish nonadherent nonhemolyzing colonies of streptococci.

Sections across the apex of the gallbladder disclosed marked fibrosis, thickening of blood vessels, atrophy of mucous membrane, hemorrhagic infiltration,

chiefly in the submucosa and the peritoneal coat, numerous eosinophils in connective tissue, and a moderate number of gram-staining diplococci in hemorrhagic areas in the submucosa and the subperitoneum (Figs. 3 and 4).

The streptococcus from the stone and that from the wall of the gallbladder were each injected, in the second generation, into 1 dog and 1 rabbit. Both dogs (100 and 101) developed marked cholecystitis, but the rabbits appeared well 48 hours after injection. The dog injected with the culture from the gallbladder had 2 small hemorrhages in the tricuspid valve, hemorrhages in the diverticulum of Vater, and 1 hemorrhage in the gallbladder. The other had 2 small hemorrhages in the tricuspid valve and a few in the subcutaneous tissue, but no lesions of the gallbladder or bile ducts. The strain from the wall of the gallbladder after cultivation on blood agar for 10 days, and then in ascites dextrose broth, was injected into 3 dogs. All three seemed well soon after injection, and were chloroformed, 2, 4, and 6 days later, respectively. Dog 128 had a few fading hemorrhages in the fundus of the gallbladder. The others had no lesions.

Fig. 4. Diplococcus in hemorrhagic area shown in Fig. 3. Gram-Weigert.  $\times 1000$ .

A 30-c.c. portion of the suspension containing pieces of tissue in the second culture, which was injected into 1 rabbit and 1 dog and which produced marked lesions of the gallbladder, was placed in the ice chest August 24, 1914, and the bacteria allowed to remain in latent life undisturbed until March 15, 1915. Blood-agar-plate cultures then contained many small nonhemolysing grayish colonies exactly as when isolated.

March 16.—Two dogs were injected, one with 3 c.c. and the other with 12 c.c. of this old suspension. Both died in 24 hours, showing marked hemorrhage and edema of the gallbladder, and hemorrhages of the stomach, duodenum, and intestinal tract. The one receiving 12 c.c. had, in addition, hemorrhage and edema of the cystic and common ducts. In the blood of both were pure cultures of a slightly green-producing streptococcus; in the bile and the edematous fluid were countless numbers of the injected streptococcus.

The streptococci in the first subculture in ascites dextrose broth from the old suspension were injected March 18 into 2 dogs. Both developed lesions in the gallbladder, one a few hemorrhages in the stomach, and the other in the pancreas. Both dogs were well when chloroformed 48 hours after injection; the blood was sterile.

A culture in ascites dextrose broth made directly from the edematous gallbladder of the dog which had been injected with 12 c.c. of the old suspension, was injected into 1 dog and 1 rabbit. Both developed marked lesions of the gallbladder. The strain from the dog's gallbladder, injected into 1 rabbit, gave rise to no lesions of the gallbladder, but gave rise to multiple arthritis. The strain from the rabbit's gallbladder was injected into 3 dogs and 2 rabbits in doses ranging from 2 to 75 c.c. All the dogs and 1 of the rabbits developed cholecystitis. Two weeks after the injection the gallbladders of 1 rabbit and 1 dog showed healing cholecystitis with distinct fibrous thickening.

The fact that such small amounts of the old suspension were so toxic and showed the same striking affinity for the gallbladder as did this strain in fresh

cultures, afforded excellent opportunity to test whether or not there was present in the streptococcus cultures a filterable virus, and whether or not streptococci from cholecystitis having marked affinity for the gallbladder produce soluble products with the power to cause lesions electively in the gallbladder. A dog injected with 2 c.c. of the old filtered suspension—which had been proved sterile by cultures in ascites dextrose broth and on blood agar—had a number



Fig. 5. Cholecystitis in a rabbit (R 188) 5 days after intravenous injection of the streptococcus shown in Fig. 8, after it had been kept in the ice-chest for 7 months and then passed through one animal (D275). Natural size.

of small hemorrhages in the peritoneal coat near the apex of the gallbladder and a few in the mucous membrane near the fundus. Cultures from one hemorrhagic area yielded a pure growth of the streptococcus, which passed through the filter in small numbers, because subsequent cultures from the centrifugated sediment of a portion showed the streptococcus. Filtrates of the first subculture of this aged suspension and of the 24-hour-broth culture, after 1 and 2 animal passages, were injected into 5 dogs and 1 rabbit; only 1 dog developed lesions of the gallbladder. Cultures from the hemorrhagic gallbladder and from the blood in this dog remained sterile, and 45 c.c. of this sterile culture from the hemorrhagic area in the gallbladder failed to produce lesions in the gallbladder on subsequent injection. A filterable virus, therefore, appears absent, but there seem to be soluble products in these young cultures which in distinctly smaller amounts than are required of similar filtrates from other streptococci, tend to produce lesions in the gallbladder when injected intravenously.

The piece of tissue added originally to the dextrose broth and contained in the suspension which was kept in the ice chest 9 months, was preserved in the bottom of a test tube in the ice chest until March, 1916, 20 months after the cultures had been made. Subcultures from this were now made on blood agar and into ascites dextrose broth. One ringtail monkey, 1 dog, and 1 rabbit were injected with the growth in ascites dextrose broth. All seemed well 72 hours after injection, when they were chloroformed. All had developed lesions of the gallbladder (Fig. 6) but practically no lesions in other organs. The streptococcus was found in the hemorrhagic area in moderate numbers (Fig. 7).

*Dog 100.*—Injected intravenously, August 24, with the growth from 45 c.c. of an ascites-dextrose-tissue-broth culture.



Figure 6

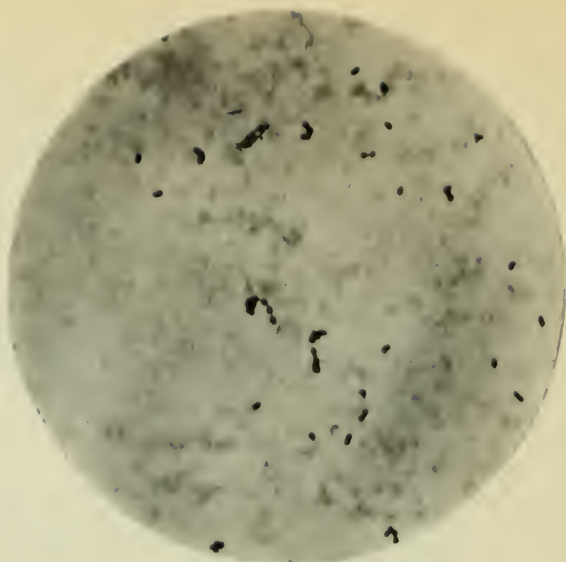


Figure 8

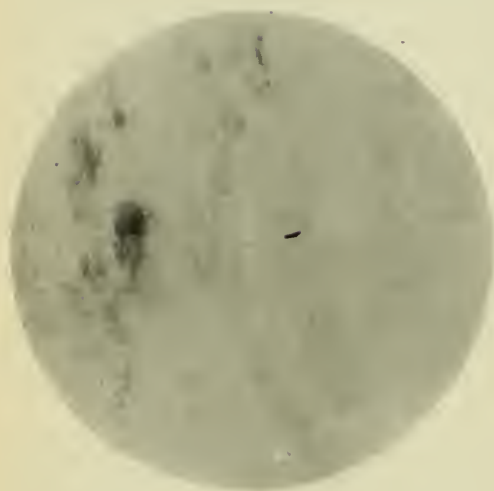


Figure 7



Figure 9

Fig. 6. Gallbladder in a dog (D 430) 72 hours after intravenous injection of a subculture of the streptococcus shown in Fig. 8, after it had been kept in the ice-chest for 20 months. About natural size.

Fig. 7. Two diplococci in the hemorrhagic area in the subperitoneum of the dog's gallbladder shown in Fig. 6. One diplococcus in partial focus. Gram-Weigert.  $\times 1200$ .

Fig. 8. A smear of a 24-hour culture in ascites dextrose broth of a streptococcus isolated from the gallbladder in Case 140. The morphology, size, and grouping are typical of the various strains isolated in cholecystitis. Gram stain.  $\times 1200$ .

Fig. 9. Hemorrhage and infiltration in the gallbladder in Dog 100, 48 hours after intravenous injection of the streptococcus shown in Fig. 8. Three-fourths natural size.

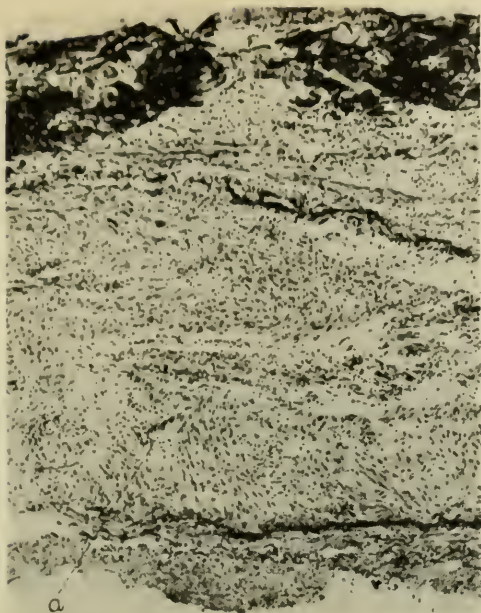


Figure 10

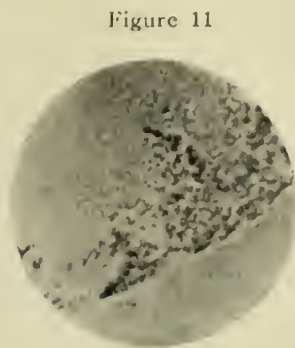


Figure 11

Fig. 10. Section of the gallbladder of Dog 101, 48 hours after intravenous injection of the streptococcus shown in Fig. 8. Marked leukocytic infiltration in the submucosa, between the muscular bundles in the muscular coat, and in the peritoneum. The latter is covered with a thick layer of exudate, rich in red blood corpuscles and leukocytes. Hematoxylin and eosin.  $\times 40$ .

Fig. 11. Diplococci in the peritoneal exudate shown in Fig. 10. Gram-Weigert.  $\times 1200$ .

Aug. 26.—Seemed well. Chloroformed. Gallbladder filled with turbid bile; the wall edematous and hemorrhagic (Fig. 9). Mucosa normal. No hemorrhages along the ducts or in the liver, and no other noteworthy lesions.

Aug. 27.—Cultures from blood, kidney, liver, bile, and joint-fluid sterile.

*Dog 101*.—Injected intravenously, August 24, with the growth from 90 c.c. of an ascites-dextrose-tissue-broth culture of the streptococcus isolated from gallbladder wall.

Aug. 26.—Seemed well. Chloroformed. Acute hemorrhagic cholecystitis. The wall of the gallbladder was covered in areas with a fibrinous exudate and contained the large subperitoneal hemorrhages, particularly over the fundus, associated with moderate edema. No noteworthy changes in the mucous membrane. Ducts free from hemorrhage. Lymph glands along the cystic and common ducts enlarged and hemorrhagic on the cut surface. Joint fluid slightly turbid.

Aug. 27.—Cultures from blood, joint fluid, bile, and liver were sterile, while those from the infiltrated wall of the gallbladder contained the streptococcus. Sections through a hemorrhagic area near the fundus of the gallbladder showed a layer of fibrinous exudate rich in leukocytes covering the peritoneal coat, leukocytic infiltration of the peritoneal and subperitoneal layer, especially in localized areas surrounding blood vessels and dilated lymph spaces (Fig. 10), and an aggregation of leukocytes in a rather large blood vessel in the subperitoneum. The mucous membrane and the aggregation of lymphoid cells in the submucosa were free from lesions and revealed no leukocytic infiltration.

A moderate number of diplococci and a few short chains were in the exudate covering the peritoneum and in the areas showing leukocytic infiltration (Fig. 11).

*Dog 128.*—Injected intravenously, September 3, with the growth from 75 c.c. of an ascites-dextrose-broth culture of the streptococcus isolated from the wall of the gallbladder 10 days previously.

Sept. 5.—Seemed well. Chloroformed. A few small hemorrhages in the mucous membrane of the stomach and a number of small fading subperitoneal hemorrhages over the fundus of the gallbladder. No noticeable associated edema or infiltration. Gallbladder distended with greenish bile and viscid mucus.

Sept. 7.—Cultures on blood agar from blood and bile were sterile, while those from the blood in dextrose broth yielded a pure culture of the streptococcus.

#### CASE 166

A young man, with acute cholecystitis and pancreatitis of 10 days' duration, was operated on Oct. 12, 1914. A markedly edematous pus-containing gallbladder was found, embedded in a mass of fibrinous adhesions. The pus from the lumen and a portion of the wall of the gallbladder were removed and cultured. Smears from the former showed what appeared to be colon bacilli and streptococci.

Oct. 13.—Cultures from the wall of the gallbladder and from the pus gave 3 varieties of colonies, one of which appeared to be colon bacilli, another *B. mucosa*, and a third smaller and more opaque variety resembled staphylococci.

Oct. 15.—Subcultures on Loeffler's serum of the staphylococcus revealed a moderate amount of gray growth. The mucoid colonies were made up of strictly aerobic encapsulated gram-negative nonmotile bacilli, which did not produce gas in dextrose-agar stabs, while the colon-bacillus-like colonies showed typical nonencapsulated motile gas-producing bacilli.

A suspension in NaCl solution of pus failed to produce lesions in the one dog injected. A mixture of the staphylococcus and of *B. mucosus* injected into 1 dog produced numerous hemorrhages in the stomach, the small intestine, and the medulla of the kidney, and acute hemorrhagic pancreatitis with fat necrosis, while the one rabbit which was injected died with marked hemorrhages in the stomach and the intestine. The staphylococcus after 1 animal passage was injected into 2 dogs and 1 rabbit. Both dogs had pancreatitis and 1 had cholecystitis, while the rabbit had endocarditis and myocarditis.

*Dog 190.*—Injected intravenously, Oct. 15, with the growth from 15 c.c. of an ascites-dextrose-broth culture of the staphylococcus and the encapsulated bacillus mixed.

Oct. 16.—Found dead, body warm. Numerous small hemorrhages and erosions in the mucous membrane of the stomach, marked enteritis, and marked hemorrhages in the medulla of the kidney. Pancreas hemorrhagic and studded with numerous irregular white areas of fat necrosis.

Oct. 17.—The blood and the pancreas showed staphylococci in pure culture.

*Dog 195.*—Injected intravenously, Oct. 21, with the growth from 5 c.c. of an ascites-dextrose-broth culture of the staphylococcus after 2 animal passages.

Oct. 23.—Seemed ill.

Nov. 6.—Seemed well but had lost in weight. Chloroformed. Pancreas swollen, hard, and hyperemic. Bile thick and dark, containing mucus in which were embedded numerous black particles. On the wall of the gallbladder was an opaque band of fibrous thickening, running from the apex over the fundus.

Nov. 11.—Cultures from the bile on dextrose agar yielded a large number of gram-staining cocci singly, in twos, and in groups. The blood was sterile.

## CASE 230

A woman, 32 years of age, was operated on Dec. 30, 1914. Gallbladder edematous and distended with mucopurulent material, in which were embedded numerous small stones. Chills and fever, intense pain and tenderness over the gallbladder had begun 5 days before the operation. There had been 4 milder attacks during the previous 9 months.

Jan. 2.—In the aerobic cultures from scrapings from the wall of the gallbladder, from the stone, and the pus, were colon bacilli only, while in the anaerobic cultures there were colon bacilli and *B. fusiformis*.

Two dogs and 2 rabbits were injected with the growth from ascites dextrose broth containing colon bacilli only; both dogs and 1 rabbit developed marked cholecystitis. The rabbit and 1 dog had, in addition, lesions of the cystic and common ducts. The other rabbit had small hemorrhages in duodenum, stomach, and appendix. In 1 dog, injected intravenously with the anaerobic culture on blood agar, a marked diffuse cholecystitis and cholangitis were produced. The dog in which an injection was made directly into the gallbladder developed only a localized edema of the gallbladder and peritonitis around the point of puncture. The colon bacillus isolated from the edematous gallbladder of Dog 204, produced marked hemorrhagic enteritis and pancreatitis in the one dog injected.

*Dog 204.*—Injected intravenously, Dec. 31, with the growth from 45 c.c. of an ascites-dextrose-broth culture from the pus.

Jan. 2.—Found dead. Gallbladder markedly edematous; 0.7 cm. in thickness, and covered with a hemorrhagic fibrinous exudate. Two areas had become gangrenous. In smears from the edematous fluid were a large number of colon bacilli. In the liver were areas of hemorrhage; the mucous membrane of the intestinal tract was hemorrhagic; the intestinal contents were bloody; and the pericardial sac contained bloody fluid. Sections showed marked hyperemia of

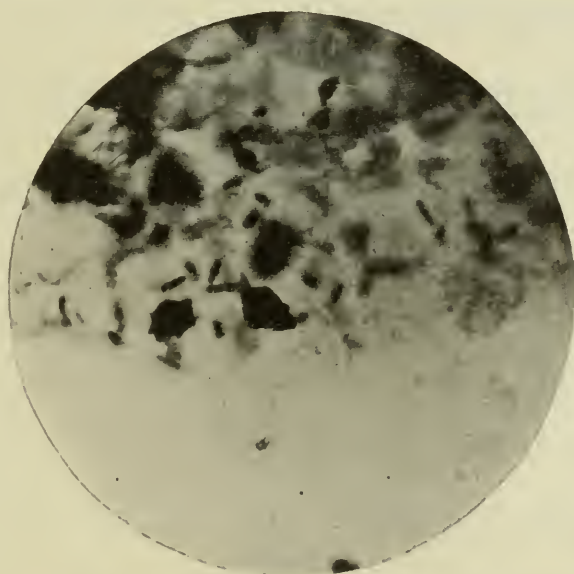


Fig. 12. Colon bacilli in the wall of a markedly edematous gallbladder (D 104) 24 hours after intravenous injection of colon bacilli from acute cholecystitis in man. Gram-Weigert (partial decolorization).  $\times 1200$ .

TABLE 1  
SUMMARY OF THE RESULTS OF CULTURES IN CHOLECYSTITIS

Case	Sex and Age	Anatomic Diagnosis	Duration of Symptoms	Results of Cultures From	
				Bile or Other Fluid Contents of Gallbladder	Gallstones
41	.. ..	Cholecystitis with stones	.....	.....	Colon bacillus
55	.. ..	Chronic cholecystitis with stones	.....	.....	Streptococcus (one colony)
58	F 50	Chronic cholecystitis with stones and appendicitis	20 years	Streptococcus and colon bacillus	Streptococcus.....
59	F 56	Chronic cholecystitis and chronic appendicitis	2 or 3 years	.....	.....
60	F 36	Chronic catarrhal cholecystitis	10 years	Negative.....	.....
61	F 59	Chronic cholecystitis with stones	15 years	Sterile.....	Streptococcus.....
66	M 75	Chronic cholecystitis.....	9 years	Streptococcus and colon bacillus	.....
67	M 56	Chronic cholecystitis.....	10 years	.....	.....
68	F 50	Chronic cholecystitis with stones	16 years	.....	Streptococcus and B. Welchii
70	F 28	Chronic cholecystitis with stones	2 years	Negative.....	Streptococcus (3 colonies)
73	M 52	Chronic catarrhal cholecystitis and appendicitis	12 years	Negative.....	.....
79	M 53	Chronic cholecystitis with stones	24 years	Staphylococcus....	Streptococcus.....
85	M 60	Cholecystitis and pancreatitis	5 months	Streptococcus, colon bacillus, and fusiform bacillus	Streptococcus and colon bacillus
91	M 48	Gangrenous gallbladder and encysted stone	9 months	.....	Streptococcus (60 colonies)
93	M 32	Pancreatitis, cholecystitis and appendicitis obliterated	.....	Negative.....	.....
94	M 58	Cholecystitis, probably secondary to perforating duodenal ulcer	20 years	Colon bacillus.....	Colon bacillus and streptococcus
98	F 50	Chronic cholecystitis with stones	12 years	Streptococcus and diphtheroid bacillus	Streptococcus.....
99	M 24	Chronic cholecystitis.....	5 months	Negative.....	.....
101	F 41	Chronic cholecystitis with stones	2½ years	Colon bacillus.....	Streptococcus and B. Welchii
102	M 55	Chronic catarrhal cholecystitis	6 years	Colon bacillus.....	.....
108	F 52	Chronic cholecystitis with stone	25 years	.....	Streptococcus.....
115	M 44	Chronic cholecystitis with stones	3 years	Negative.....	Negative.....
117	F 45	Acute cholecystitis with common duct stone	4 or 5 years	Colon bacillus.....	Streptococcus.....
120	M 55	Cholecystitis with stone and duodenal ulcer	7 years	Negative.....	Streptococcus, colon bacillus, and B. Welchii

TABLE 1—Continued  
SUMMARY OF THE RESULTS OF CULTURES IN CHOLECYSTITIS

Results of Cultures From		Remarks
Gallbladder Wall	Adjacent Lymph Glands	
.....	.....	.....
Streptococcus (4 colonies)	.....	.....
Streptococcus.....	.....	Cultures from obliterated appendix, sterile
Streptococcus and colon bacillus	.....	Cultures from appendix, streptococcus and colon bacillus
Negative.....	.....	Gallbladder showed doubtful lesions
Streptococcus (27 colonies) and B. Welchii	Streptococcus (5 colonies)	Cultures made from infarcted area near fundus of gallbladder
Streptococcus and colon bacillus	.....	Gallbladder had been drained for 3 years
.....	Streptococcus and diphtheroid bacillus	..
.....	.....	.....
Streptococcus.....	.....	Strawberry gallbladder
Negative.....	.....	Thickening of gallbladder slight. Wall of appendix showed colon bacillus and streptococcus
Negative.....	.....	Gallbladder contracted tightly on single stone
Streptococcus and colon bacillus	.....	Large amount of thick tenacious dark material in gallbladder, in which was imbedded much "sand" and a number of calculi, size of wheat grains
Streptococcus, colon bacillus, and fusiform bacillus	.....	First attack of cholecystitis in which stone was probably formed, 10 years before. Second attack in which stone became encysted, 3½ months before
Streptococcus and diphtheroid bacillus	.....	Tip of appendix, portion just beyond obliterated lumen, showed colon bacillus and streptococcus
Streptococcus (3 colonies)	.....	Ulcer had probably existed for 20 years
Streptococcus (120 colonies)	.....	Symptoms of cholecystitis for 12 years. Wall of gallbladder thickened, mucous membrane destroyed. Many large and small stones
Streptococcus or diphtheroid bacillus	.....	Wall of gallbladder moderately thickened
.....	.....	Wall of gallbladder only slightly thickened
Colon bacillus.....	.....	Gallbladder only slightly thickened
Streptococcus and colon bacillus	.....	Typhoid fever 30 years before; symptoms referable to stomach ever since, worse recently. Gallbladder wall thickened
Staphylococcus...	.....	Symptoms suggestive of appendicitis. No evidence of recent inflammation of gallbladder. Wall not much thickened. Stones small
B. Welchii and colon bacillus	B. Welchii and colon bacillus	Patient had chills and fever at time of operation, which promptly disappeared afterward
Streptococcus (6 colonies)	Streptococcus (one colony)	Duodenal ulcer markedly indurated with partial obstruction to pylorus. Gallbladder and duodenum adherent

TABLE 1—Continued  
SUMMARY OF THE RESULTS OF CULTURES IN CHOLECYSTITIS

Case	Sex and Age	Anatomic Diagnosis	Duration of Symptoms	Results of Cultures From	
				Bile or Other Fluid Contents of Gallbladder	Gallstones
122	F 49	Chronic catarrhal cholecystitis with stones	17 years	Negative.....	Negative.....
124	F 39	Chronic cholecystitis with stones	22 years	Streptococcus and colon bacillus	B. Welchii.....
128	F 68	Chronic catarrhal cholecystitis with stones	.....	Negative.....	.....
132	M 38	Chronic appendicitis and catarrhal cholecystitis	14 years	Negative.....	.....
135	F 68	Acute and chronic cholecystitis with stones	15 years	Streptococcus and colon bacillus (many colonies, mostly colon bacillus)	Streptococcus and colon bacillus (many colonies, mostly colon bacillus)
138	F 39	Chronic cholecystitis with stones	5 years	.....	Streptococcus (one colony)
140	F 34	Acute and chronic cholecystitis with stones	5 or 6 years	Negative.....	Streptococcus (7,200 colonies)
166	M ..	Acute cholecystitis and pancreatitis	.....	Colon bacillus, B. mucosus, and staphylococcus	.....
168	F ..	Empyema of gallbladder with stones	.....	Sterile.....	Streptococcus.....
230	F 32	Subacute cholecystitis. Empyema of gallbladder	.....	Colon bacillus and fusiform bacillus	.....
494	F 41	Chronic catarrhal cholecystitis with stones	.....	.....	Streptococcus and B. Welchii
497	F 43	Chronic cholecystitis with stones	.....	.....	.....
500	F 35	Chronic catarrhal cholecystitis with stones	.....	.....	Diphtheroid-like streptococcus
503	F 60	Subacute cholecystitis with stones	.....	Streptococcus and colon bacillus	Negative
523	F 37	Chronic catarrhal cholecystitis. Chronic catarrhal appendicitis	.....	.....	.....
570	F 18	Chronic catarrhal cholecystitis with stones	.....	Sterile.....	B. subtilis (2 colonies, contamination)
576	F 54	Chronic cholecystitis with stones. Appendicitis obliterated	.....	.....	.....
869	F 62	Subacute cholecystitis with stones	.....	Sterile.....	Streptococcus, colon bacillus, B. Welchii
971	.. ..	Cholecystitis.....	.....	.....	Streptococcus
977	.. ..	Cholecystitis with stones..	.....	.....	Streptococcus
984	.. ..	Cholecystitis with stones..	.....	.....	Unidentified gram-positive spore-bearing bacillus and typhoid bacillus
988	.. ..	Cholecystitis with stones..	.....	.....	Streptococcus
999	.. ..	Cholecystitis with stones..	.....	.....	Streptococcus
D	.. ..	Cholecystitis with stones..	.....	.....	Negative.....

TABLE 1—*Continued*  
SUMMARY OF THE RESULTS OF CULTURES IN CHOLECYSTITIS

Results of Cultures From		Remarks
Gallbladder Wall	Adjacent Lymph Glands	
Negative.....	.....	Gallbladder distinctly thickened
Streptococcus and colon bacillus	.....	Mucous membrane destroyed. Wall much thickened
.....	.....	Gallbladder wall slightly thickened. Stone removed and gallbladder drained
Streptococcus (one colony)	.....	Gallbladder only slightly thickened
Streptococcus and colon bacillus (many colonies, mostly colon bacillus)	Streptococcus	.....
Negative.....	.....	Only slight thickening of gallbladder. Typical strawberry gallbladder
Streptococcus (40 colonies)	.....	Symptoms of cholecystitis and stone began 5 years before. Present attack began 20 days before. Pain severe. Gallbladder filled with pus and gallstones
Colon bacillus and staphylococcus	.....	Edematous omentum showed colon bacillus
.....	.....	.....
Colon bacillus, B. mucosus, and fusiform bacillus	.....	.....
.....	.....	Strawberry gallbladder
Streptococcus and colon bacillus	.....	Distal 6th of lumen of appendix obliterated
.....	.....	Strawberry gallbladder. Fibromyomata of uterus. Chronic salpingitis and ovaritis
Streptococcus (5,000 colonies) and colon bacillus (few colonies)	.....	.....
.....	Colon bacillus....	Strawberry gallbladder. Obliteration of mucosa at distal end of appendix
Diphtheroid bacillus	Sterile.....	Distal third of lumen of appendix obliterated
.....	Streptococcus (12 colonies)	.....
Streptococcus....	.....	.....
.....	.....	Cultures made 1 year after stones were removed

vessels, hemorrhage, leukocytic infiltration, edema, poorly staining nuclei of the fixed tissue cells, and numerous colon bacilli (Fig. 12).

Jan. 4.—Cultures from the bile, wall of the gallbladder, liver, and pericardial fluid gave colon bacilli in pure growth.

*Dog 205*.—Injected intravenously, Jan. 2, with the growth from 1 blood-agar slant.

Jan. 4.—Seemed fairly well. Chloroformed. Marked edema and hemorrhage of the gallbladder and of the cystic and common ducts. Mucous membrane edematous, hemorrhagic, gangrenous in areas, and covered with a thick adherent mucus. Stomach, duodenum, and intestinal tract showed no noteworthy changes. Joint fluid clear.

RESULTS OF ANIMAL EXPERIMENTS

The foregoing details will serve to illustrate the results obtained in other cases. In Table 2 is given a summary of the animal experiments made with strains from cholecystitis.

Of animals injected with the strains when isolated, 79% developed lesions of the gallbladder. This is in marked contrast to an average

TABLE 2  
ELECTIVE LOCALIZATION OF STREPTOCOCCI FROM CHOLECYSTITIS

Source of Streptococci	Strains	Animals Injected	Percentage of Animals Showing Lesions in				
			Appendix	Stomach Hemorrhage	Duodenum Ulcer	Gall-bladder	
Cholecystitis {	As isolated.....	16	53	0	29	16	79
	Later.....	5	14	14	28	14	7
	After animal passage...	4	16	0	31	13	56

incidence of lesions in the gallbladder of 11% as previously pointed out,<sup>9</sup> and to an incidence of 3% in 396 animals following injection of bacteria from sources other than cholecystitis. Later, that is after cultivation on artificial media for a time, this affinity was found to have largely disappeared, just as it tends to disappear after successive animal passages, lesions in the gallbladder occurring in 7% and 56% of the animals respectively (Table 1). If, however, the bacteria are placed in latent life, as occurs in the single colonies in shake cultures, or in NaCl suspensions containing tissue in the ice chest, the characteristic affinity may be retained for a long time. Thus the streptococcus from Case 140 lost nearly all its affinity for the gallbladder after cultivation on blood agar for 10 days, whereas when kept in the ice chest in NaCl solution its affinity for the gallbladder was marked 8 and 20 months later.

The incidence of pancreatitis, following injection of the strains from cholecystitis as isolated, was 5% ; after cultivation 0% ; and after animal passage 19%. In some of the animals, especially those injected with strains after animal passage, there were noted simultaneously lesions of the gallbladder, bile ducts, and pancreas (Fig. 13).

Elective affinity for the gallbladder was shown by 16 strains of streptococci from cholecystitis as isolated. Most of these were isolated from the wall of the gallbladder, 3 from the centers of gallstones, and 1 from an adjacent lymph gland. Only 22% of the animals, chiefly rabbits, died from the effects of the injection; the rest were killed. The colon bacilli from 3 cases of cholecystitis with stones, in which streptococci also were isolated, showed no special affinity for the gallbladder, while 2 strains from acute cholecystitis in which streptococci were absent, showed marked affinity for the gallbladder. This suggests that they were secondary invaders in the former, and the cause of cholecystitis in the latter. The staphylococcus

TABLE 2—*Continued*  
ELECTIVE LOCALIZATION OF STREPTOCOCCI FROM CHOLECYSTITIS

Percentage of Animals Showing Lesions in									
Pan- creas	Intes- tines	Joints	Endo- cardi- um	Peri- cardi- um	Myo- cardi- um	Muscles	Kidney	Lung	Skin
5	19	17	12	0	2	9	5	8	3
0	0	21	14	0	0	0	7	0	0
19	13	25	19	0	13	0	13	6	0

from an acute case showed marked affinity for the gallbladder. The lesions following injection of the colon bacillus and staphylococcus were not confined so strikingly to the gallbladder as in the case of the streptococcus. Mixed cultures in ascites dextrose broth of streptococci and colon bacilli from the gallbladder in cholecystitis were injected directly into 11 animals; of these, 5 (46%) developed lesions of the gallbladder, while the appendix and stomach showed no noteworthy lesions. These findings are again in marked contrast to those following injections of the primary mixed cultures of streptococci and colon bacilli from appendicitis and ulcer of the stomach. The mixed cultures from appendicitis produced lesions of the appendix in 30 (78%) of 37 rabbits, of the stomach or duodenum in 9, and of the gallbladder in 4. Those from ulcer produced lesions of the stomach or duodenum in 5 (62%) of 8 animals, of the gallbladder in 1, and of the appendix in

none. The tendency to infect the tissues in animals corresponding to the diseased tissues from which the organisms were isolated is shown, therefore, even by mixed cultures. It should be stated, however, that in most of these the streptococcus determined the elective localization.

The elective localization of the bacteria from the tonsils was tested in the case of 3 patients who had recurring attacks of cholecystitis. The cultures from 2, one during an acute exacerbation, the other 3 days after cholecystectomy, were injected into 2 dogs and 4 rabbits. Both dogs and 2 of the rabbits developed striking lesions in the gallbladder, the rabbits showing lesions in the muscles in addition; the other 2 rabbits had ulcer of the stomach. The cultures from the tonsils of the 3rd patient, 1 week after an acute attack, did not produce lesions of the gallbladder, but produced myositis in 1 dog and 1 rabbit.

In a summary of the cultures from animals with lesions in the gallbladder following injection of streptococci, it was found that the streptococci were isolated from the blood at the time of autopsy in only 16 of 62 animals; from the bile in 14 of 66; from the wall of the gallbladder in 15 of 21; and from the joint-fluid in 6 of 38. In 3 instances cultures from the bile or wall of the gallbladder showed a secondary invasion of colon bacilli (Case 120, Dog 114). The number of colonies from the wall of the gallbladder, the seat of experimental cholecystitis, ranged from a few to 8,000.

The strains of streptococci from myositis, ulcer of the stomach, and after one or more animal passages, were especially prone to cause lesions in the gallbladder (Figs. 15, 16, 17, 18, 20, and 22).

Definite formation of gallstones, resembling in appearance those found in one of the patients in this series, was noted altogether in 6 rabbits and 3 dogs with cholecystitis (Fig. 2). In some of these animals, streptococci were present in the gallstones in pure form when absent in the surrounding fluid and salt-solution washings.

The milder lesions in experimental cholecystitis consisted of circumscribed areas of subperitoneal hemorrhage, fibrinous deposit on the peritoneum, aggregation of leukocytes in and surrounding blood vessels (Fig. 10), while the marked changes often consisted of extreme edema from 24 to 48 hours after injection chiefly of strains from acute cholecystitis. In the former there was often marked leukocytic infiltration (Fig. 10), but in the latter this was usually absent. The nuclei of the cells in fixed tissue stained faintly, and the mucous membrane appeared necrotic (Fig. 16). Sections of these gallbladders cut transversely at the apex often showed extreme dilatation of lymph vessels and tissue



Fig. 13. Hemorrhages in the gallbladder (a), common duct (b), and head of the pancreas (c), in a dog (D 144) 4 days after intravenous injection of a streptococcus isolated from ulcer of the stomach in man (Case 52), after 1 animal passage. Natural size.



Fig. 14. Localized hemorrhagic cholecystitis and hepatitis in a dog (D 139) 3 days after intravenous injection of a streptococcus from duodenal ulcer in man (Case 112). Natural size.



Fig. 15. Edema of the gallbladder in a dog (D 142) 24 hours after injection of the streptococcus from the gallbladder shown in Fig. 14. Natural size.

spaces, which were filled with edematous fluid (Fig. 16). In size the arteries usually appeared normal or distinctly dilated.

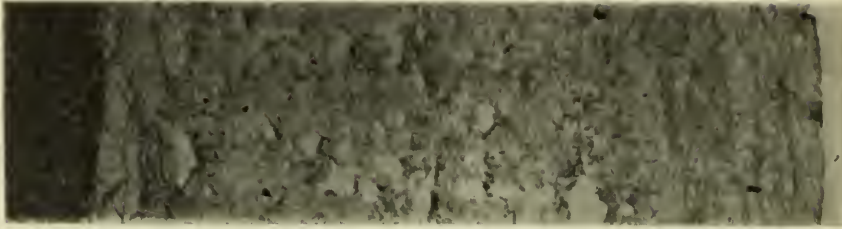


Fig. 16. Cross section of the gallbladder in a dog (D 63) 9 days after intravenous injection of a streptococcus from human ulcer of the stomach and cholecystitis (Case J). Extreme edema, dilatation of the lymph channels and connective tissue spaces, and almost complete absence of staining of nuclei. The stomach of this dog showed 2 ulcers near the pyloric end, and the mucous membrane of the gallbladder was ulcerated in several areas. Hematoxylin-eosin.  $\times 20$ .

There was a striking difference between the location of lesions in the gallbladders of dogs following injection of streptococci, and that following injection of colon bacilli. Following the former, the lesions were chiefly in the peritoneal and muscular coat, the mucous membrane being involved secondarily and only when the lesions were marked; following the latter, the mucous membrane was often necrotic or sloughed, and the peritoneal and muscular coat involved but slightly.

The demonstration of bacteria both in cholecystitis in man and in the experimental form of the disease was difficult, even where the cultures showed large numbers of colonies. This appeared to be especially true when the edematous fluid was stained with bile. In some of these colonies the bacteria appeared swollen and partially digested (Fig. 17).

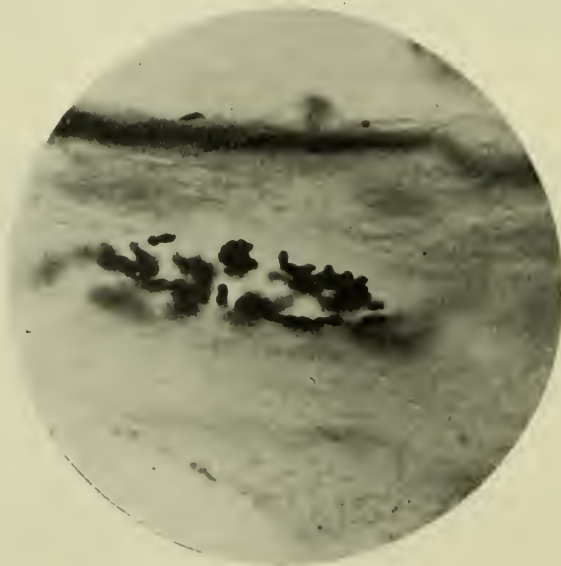


Fig. 17. Numerous swollen diplococci in a lymph channel beneath the peritoneum in the edematous gallbladder shown in Fig. 15.  $\times 1200$ .

## THE STREPTOCOCCI FROM CHOLECYSTITIS

All strains produced acid in dextrose and maltose, and none produced acid in inulin, or in the controls of the sugar-free broth; hence these are omitted in Table 3. The broth was prepared in the standard way from beef infusion, 1% of the various test substances added, the resultant media rendered 0.6% acid to phenolphthalein, sterilized frac-

TABLE 3  
FERMENTATIVE REACTIONS OF STREPTOCOCCI FROM CHOLECYSTITIS

Date	Strain	Lactose	Saccharose	Raffinose	Mannite	Salicin
6/ 7/15	140	+++	+++	0	+++	+++
3/17/15	140	—	+++	0	+++	+++
3/28/16	140	0.7	0.4	0.2	1.9	2.5
1/21/15	140 <sup>2</sup>	—	..	—	—	+
12/21/15	140 <sup>2</sup>	0	0	0	0	1.5
6/ 7/15	55	+	++	0	0	0
	58	0	..	0	++	++
12/20/15	58	0.3	1.5	0	0.8	1.8
	58*	+	..	0	++	++
	85	++	..	0	++	++
12/21/15	85	1.4	0	0	1.1	2.0
	85 <sup>2</sup>	++	..	0	++	++
	61	++	..	+	0	++
	91	++	..	0	++	++
1/21/15	91	+	..	—	+	+
12/20/15	91	1.6	1.3	1.0	0	1.9
	91*	+	..	0	++	++
1/21/15	91*	+	..	—	+	+
3/28/16	91*	1.1	2.7	0.1	2.2	2.6
	93	++	..	0	++	++
1/21/15	93	+	..	—	+	+
1/21/15	135*	+	..	—	+	+
	135*	+	..	0	++	++
12/20/15	135*	0.3	1.0	0	0.9	1.8
	135*	++	..	0	++	++
1/21/15	135 <sup>2</sup>	+	..	—	+	+
12/20/15	135 <sup>2</sup>	0.3	1.5	0	1.0	1.8
2/ 5/15	999*	+++	..	0	..	+++
2/ 3/16	999*	0.9	1.5	0.2	1.2	2.0

Figures to the right and above the numbers indicating strains represent the number of animal passages, the asterisk (\*) that the strains are isolated from the centers of gallstones. The rest of the strains were isolated from the walls of gallbladders. The + signs indicate roughly the degrees of acidity. The figures indicate the number of cubic centimeters N/10 sodium hydrate required to neutralize 5 c.c. of the broth culture (phenolphthalein).

tionally, and incubated for 72 hours at 37 C. The number of + signs indicates roughly the degrees of acidity, with litmus or fuchsin as indicators. The figures indicate the number of cubic centimeters N/10 sodium hydrate required to neutralize 5 c.c. of broth culture (phenolphthalein). Unless otherwise indicated in Table 3, the strains were isolated from the wall of the gallbladder itself. Altogether, 9 strains have been studied at intervals extending over a period of 11½ years. These include streptococci both from the wall of the gallbladder and from gallstones in 2 cases (Cases 58 and 91), and 3 strains before and after 1 animal passage. Lactose and salicin were fermented by all but 1



Fig. 18. Cholecystitis in a rabbit (R 407) 72 hours after intravenous injection of streptococci from ulcer of the stomach in a sheep (S 425). Natural size.

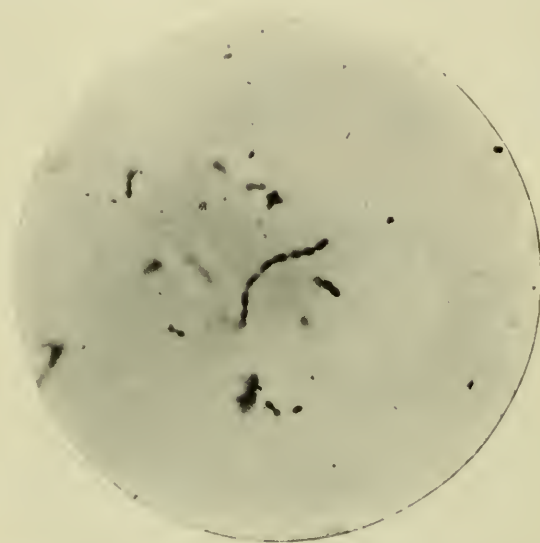


Fig. 19. Streptococci in the gallbladder of a rabbit (R 521) developing cholecystitis 6 days after intravenous injection of a streptococcus originally isolated as a pneumococcus. Strain 1.70.<sup>22</sup>  $\times 1200$ .

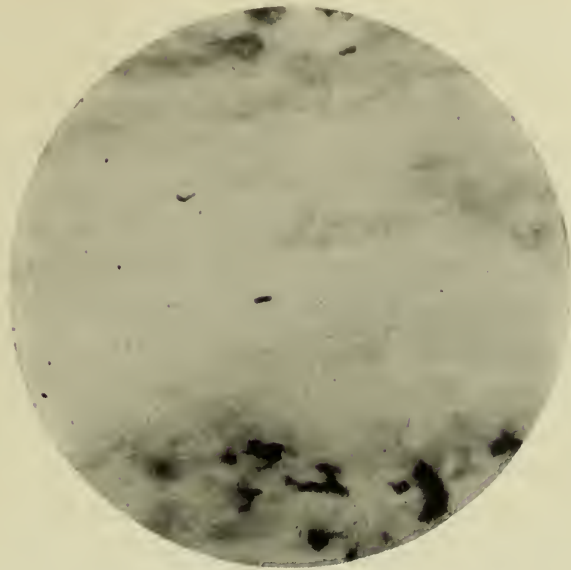


Fig. 20. Diplococci in the wall of a hemorrhagic area in the gallbladder of a dog (D 170) 24 hours after intravenous injection of a streptococcus from the thyroid gland in exophthalmic goiter (Case 80), after 1 animal passage. Gram-Weigert.  $\times 1200$ .

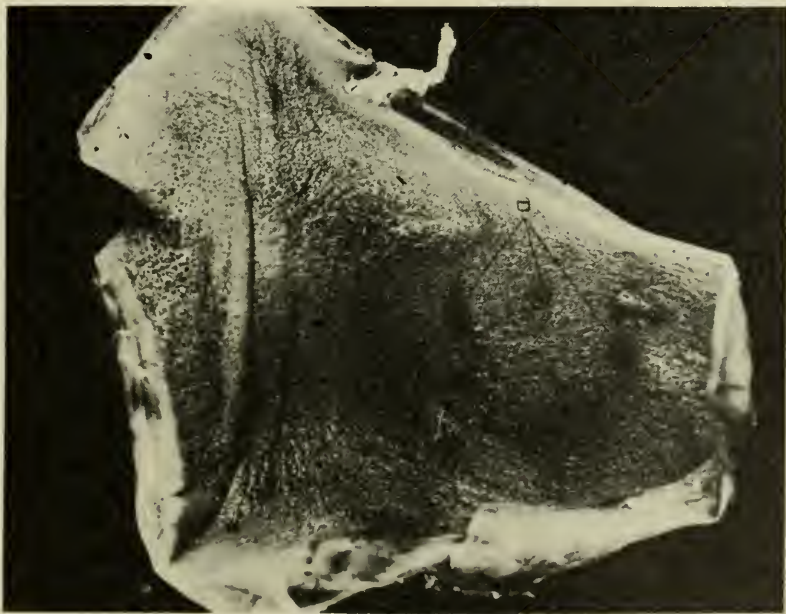


Fig. 21. Hemorrhagic and gangrenous cholecystitis in a dog (D 175) 24 hours after intravenous injection of a streptococcus from the thyroid gland in exophthalmic goiter (Case 80), after 2 animal passages. The areas of beginning gangrene are shown at A. Natural size.

strain, mannite by all but 4, saccharose by all but 2, while raffinose was fermented only by 2. The fermentative powers of strains from the centers of gallstones and from the corresponding walls of the gallbladders were identical; the fermentative powers of 1 strain only (Case 91), did not correspond on the different dates. Two of the strains after 1 animal passage showed the same fermentative powers as before (Cases 85 and 135), while 1 strain (Case 140) failed to ferment mannite after 1 animal passage.

The strains from the different cases showed a striking similarity and resembled closely the streptococci commonly found in ulcer of the stomach and appendicitis. They produced small moist grayish-brown or grayish-green nonadherent nonhemolyzing colonies on human blood agar; they produced short chains in liquid media with clumps of cocci somewhat resembling staphylococci (Fig. 8); they were of a rather low virulence, yet somewhat more virulent than the strains from gastric ulcer and appendicitis. Five of the strains had acquired distinct hemolytic powers from prolonged artificial cultivation on human blood agar. After animal passage they tended to produce more green on blood-agar plates. The loss of the power to infect the gallbladder electively occurred usually without demonstrable morphologic or cultural changes.

#### SUMMARY AND GENERAL DISCUSSION

The results thus detailed were obtained over a period of 2 years, in different localities, during different seasons of the year, and in different species of animals. The number of animals injected with some of the strains was sufficiently large to prove beyond doubt the elective property of strains isolated. Streptococci and colon bacilli from acute cholecystitis gave rise to more marked lesions in the gallbladders of dogs than did those from chronic cholecystitis. In some strains the virulence could be diminished by cultivation or increased by animal passage, and the affinity for the gallbladder still retained. Usually, however, this affinity was lost. Strains grown on artificial media acquired distinctly greater affinity for the stomach, and appendix; those passed through animals acquired affinity for the pancreas.<sup>11</sup>

In Case 120, ulcer was produced in animals with streptococci isolated from the lymph gland draining the duodenal ulcer, and cholecystitis was produced with streptococci from the gallbladder. These results furnish experimental evidence that ulcer and cholecystitis in the same patient at times may be due to hematogenous infection by streptococci which have these respective powers of localization. A single

strain, however, may have affinity for both structures, as shown in numerous experiments.

The simultaneous occurrence of lesions in the gallbladder and in the cystic and common ducts following injection of the streptococcus from the gallbladder (Case 135) where these structures were involved, indicates that the lesions may be due to the wide range of affinity of the infecting micro-organism. The occurrence at the same time of cholecystitis and pancreatitis in the animals injected with bacteria from acute cholecystitis and pancreatitis (Case 166), and with strains from chronic cholecystitis and from ulcer after animal passage (Fig. 13), suggests that the simultaneous presence of the diseases in the same patient is due commonly in the beginning to hematogenous infections and not so often to lymphogenous or local invasion as the findings at operation so often appear to indicate.<sup>12</sup>

The demonstration of streptococci in the involved tissues in a high percentage of cases of chronic cholecystitis, the elective affinity of these organisms for the gallbladder in animals, and the production of the disease with the strains isolated from the experimental lesions, indicate that streptococci are a cause of cholecystitis. The importance not only of draining but of removing gallbladders is apparent, especially in the absence of stones, as already emphasized by Mayo<sup>13</sup> and others. This is especially true in cases of chronic cholecystitis. Gallbladders removed in chronic cholecystitis during the quiescent interval have been demonstrated to be the host of living bacteria. Might not the periodic exacerbations be due to resumption of activity on the part of a latent infection, when the defensive mechanism of the individual is low? The fact that streptococci which produced marked cholecystitis by systemic intravenous injection, failed to produce cholecystitis when injected into the radicles of the portal vein, or when injected directly into the gallbladder, is crucial evidence that cholecystitis in the absence of stones is very frequently hematogenous infection, and rarely the result of invasion from the bile.

The results of differential cultures in both spontaneous and experimental cholecystitis show that the colon bacillus is commonly a secondary invader in an infection with streptococci or in a mechanical injury produced by previously formed gallstones. That it is the primary cause of cholecystitis in some instances is quite certain, because one of a series of strains of the colon bacillus from cholecystitis was present in pure culture and showed elective affinity for the gallbladder in ani-

mals (Case 230; Fig. 12). In one case, cholecystitis which had begun during convalescence in typhoid fever was proved to be due to streptococci, hence it would seem that cholecystitis occurring in typhoid fever is not always due to the typhoid bacillus.

The common presence of bacteria in the centers of gallstones, the formation of gallstones in association with cholecystitis following injection of streptococci as observed in 9 instances, and the presence of the streptococci in the newly formed stones, emphasize anew the important rôle which infection plays in the etiology of gallstones.<sup>14</sup> The almost complete absence of bacteria in the 4 pure cholesterin stones is in accord with the views of Aschoff,<sup>15</sup> Henes,<sup>16</sup> and others, who emphasize the importance of a high cholesteremia as a causative factor in the formation of gallstones. Streptococci have been isolated from the walls of gallbladders and demonstrated there in cases of chronic cholecystitis without stones. It would appear, therefore, that for the formation of gallstones 2 factors are usually necessary: (1) infection furnishing the nucleus for the precipitation of bile salts, etc., and (2) a concentrated bile of high cholesterol content.

#### BIBLIOGRAPHY

1. Blachstein: Bull. Johns Hopkins Hosp., 1891, 2, p. 96.
2. Welch: Ibid., p. 121.
3. Cushing: Ibid., 1899, 10, p. 166.
4. Koch: Ztschr. f. Hyg. u. Infektionskrankh., 1908-9, 62, p. 1.
5. Gay and Claypole: Arch. Int. Med., 1913, 12, p. 613.
6. Nichols: Jour. Exper. Med., 1914, 20, p. 573.
7. Rosenow: Jour. Infect. Dis., 1914, 14, p. 1.
8. Rosenow: Jour. Am. Med. Assn., 1914, 63, p. 1835.
9. Rosenow: Ibid., 1915, 65, p. 1687.
10. Rosenow: Ibid., 1914, 63, p. 903.
11. MacCarty: Ann. Surg., 1910, 51, 651.
12. Rosenow: Jour. Ind. State Med. Assn., 1915, 8, p. 458.
13. Mayo: Am. Jour. Med. Sc., 1914, 147, p. 469.
14. Mignot: Arch. gén. de méd., 1898, 2, pp. 129, 263.
15. Aschoff: Wien. klin. Wchnschr., 1911, 24, p. 559.
16. Henes: Jour. Am. Med. Assn., 1914, 63, p. 146.

# A MODIFICATION OF ROEMER'S INTRACUTANEOUS METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF DIPHTHERIA ANTITOXIN IN BLOOD SERA \*

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A convenient, practical, and economical test for the determination of the diphtheria-antitoxin content of blood sera is important for a number of reasons, among which may be mentioned the desirability of estimating quantitatively the efficiency of active immunization with toxin-antitoxin, the development of an active immunity after an attack of diphtheria, the antitoxin content of the blood at varying intervals after a single therapeutic or prophylactic injection of diphtheria antitoxin, the effect of one injection of antitoxin on the duration of immunity conferred by a second injection, etc. Such determination of the antitoxin content would help to give us a solid scientific foundation in many experimental and clinical problems connected with the subject of diphtheria.

The older subcutaneous test of Ehrlich for determining the antitoxin content by the death of the guinea-pig has been universally accepted as a standard, but it cannot be used with any degree of accuracy when less than 1/20 of a unit of antitoxin to the cubic centimeter of serum is to be determined. The subcutaneous test requires a large amount of serum for each test, especially when the test is made for a fraction of a unit of antitoxin. This amount is not always obtainable in the case of human beings, especially when we are dealing with small children. In addition, the subcutaneous test is expensive in that it requires the use of one guinea-pig for each test. A method, therefore, that would enable us accurately to determine as little as 1/200 unit of antitoxin, require but a small amount of serum, and be saving of animals, would be of considerable advantage. When a larger series of sera is to be tested for small amounts of antitoxin, the intradermal method is practically the only feasible one.

Recent work in active immunization against diphtheria<sup>1</sup> has shown successful results in producing an antitoxic immunity in 90-95% of

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<sup>1</sup> Park and Zingher: Jour. Am. Med. Assn., 1915, 65, p. 2216. Am. Jour. Pub. Health, 1916, 6, p. 431.

susceptible individuals. This was shown by negative Schick reactions in individuals who had previously given positive reactions. The negative Schick reaction, however, indicates only that the individual has become immune by the development of  $1/30$  of a unit (according to our work  $1/100$  unit) or more of antitoxin to the cubic centimeter; it does not show the exact amount of antitoxin that has been produced. Altho clinically it is possible to demonstrate the continuation of the immunity by repeating the Schick test at definite intervals, yet for estimating the duration of immunity it is important, according to v. Behring, to establish quantitatively the amount of antitoxin produced in the actively immunized persons.

In 1909 Römer and Sames<sup>2</sup> published a method for testing small amounts of antitoxin in blood sera. This test was based on a previous observation made by Römer<sup>3</sup> that  $1/500$  M. L. D. of a toxin was still capable of producing a distinct local necrosis when injected intradermally into the guinea-pig.

Römer and Sames determined the necrosing dose of a toxin in the guinea-pig—for example, the amount of toxin added to one unit, or any fraction of a unit of antitoxin, which is still capable of producing a slight local necrosis. This was called the Ln dose of the toxin. They found that  $1/500$  M. L. D. of the toxin was sufficiently neutralized by  $1/40,000$  unit of antitoxin to just produce a slight local effect in the animal. This amount of antitoxin in  $1/20$  c.c. of undiluted serum (the quantity used in the test with an equal volume of toxin solution) represents  $1/2000$  unit of antitoxin to the cubic centimeter.

The method suggested by Römer and Sames and later applied by Römer and Somogyi<sup>4</sup> to the testing of concentrated antitoxic horse sera, has worked satisfactorily in their own hands, but has not given reliable results in the hands of others who have tried it repeatedly. One of the difficulties lies in their attempt to make too fine a distinction between the various lesions of closely lying tests. They claim to be able to distinguish a 5% difference in antitoxin content between the individual tests. After repeated trials this has seemed to me difficult and unnecessary, especially when we are dealing with an antitoxin content between  $1/200$  unit and 10 units to the cubic centimeter of serum. A second difficulty and an equally unnecessary point is the long time, 24 hours, that the toxin and serum are required to stand before injection into the animal. Thirty minutes has proved to be a sufficient time to allow for the full union between toxin and antitoxin,

<sup>2</sup> Ztschr. f. Immunitätsf., 1909, 3, p. 49.

<sup>3</sup> Ibid., p. 208.

<sup>4</sup> Ibid., p. 433.

even in the dilutions used in the test. A third difficulty lies in the necessity of establishing a new toxin test dose, the  $L_n$ . These factors have undoubtedly discouraged the use of a test which is inherently valuable.

The modification which I have found simple and workable in the testing of many hundreds of human and animal sera, has the following advantages:

(1) The  $L +$  of the toxin is taken as the test dose and so diluted that each cubic centimeter represents  $1/100 L +$  dose.

(2) Two test points are established with each serum, the test at which no lesion develops, and the one for twice the amount of antitoxin at which a distinct lesion appears. Within these two limits lies the antitoxin content of the serum. The first test indicates a balanced combination or a slight excess of antitoxin, while the second shows the presence of a trace of free toxin. The latter test which produces a slight local necrosis represents the antitoxin content of the serum. When the necrosis is more marked, an intermediate test is made. Example: Slight necrosis at 1 unit, no necrosis at  $1/2$  unit. Antitoxin content 1 unit. If necrosis were marked, the test would be repeated for  $3/4$  unit.

(3) It saves animals in that 4 tests can be made on each guinea-pig. It also saves time, for the tests can be interpreted in from 48 to 72 hours.

(4) As little as  $1/200$  unit of antitoxin can be determined in a serum with a fair degree of accuracy. If a distinct lesion appears at this test point, the serum is considered as having no antitoxin.

#### DETAILS OF THE TEST

(1) A standard well-ripened toxin is used, so diluted with normal salt solution that 1 c.c. represents  $1/100 L +$  dose. A fresh dilution is prepared for each set of tests. If, for example, the  $L +$  is 0.5 c.c., then that amount is diluted with 99.5 c.c. of sterile salt solution. The quantity of toxin added in the test varies with the antitoxin content for which the test is made (see Table 1).

(2) The serum to be tested is used either undiluted or diluted 1:10 (0.2 c.c. serum + 1.8 c.c. salt solution), 1:100 (0.2 c.c. of serum dilution 1:10 + 1.8 c.c. salt solution), etc. The amount of serum used in the test is always 0.2 c.c., except when the test is made for  $1/200$  unit of antitoxin; then 0.4 c.c. of undiluted serum is added to 0.2 c.c. of toxin solution.

(3) Normal salt solution is added to balance the mixture. The amount is the same as that of the toxin solution minus 0.2 c.c., which represents the serum.

(4) After the addition of toxin, serum, and salt solution, the mixture is allowed to stand for 30 minutes at room temperature before it is injected into the guinea-pig. This time is utilized in preparing and tagging the guinea-pigs.

(5) The guinea-pigs (300-350 gm.) are prepared by removing the hair from the abdomen. This is best accomplished by simply pulling it out, a central line of hair being left to divide the abdominal surface into halves. The pulling of the hair is easily and rapidly carried out, and is probably less painful than the action of a depilatory like calcium hydrosulfid or shaving of the hair. The first occasionally leads to local eczema, the second to abrasions that interfere with the reading of the test.

(6) The animal holder is simply an elevated board with 4 openings for loops of cord with which to fasten the limbs of the animal. This simple device is essential in accurate work and facilitates the exact placing of the injections.

(7) A 1-c.c. "Record" or tuberculin syringe and a fine steel or platinum-iridium needle are necessary.

(8) Four injections are made into each guinea-pig, the abdomen being for this purpose divided into right upper (R. U.), right lower (R. L.), left upper (L. U.), and left lower (L. L.) quadrants. The injections are made as far apart as possible to avoid a fusion of the lesions. Two-tenths cubic centimeter of each test mixture is injected intradermally. Care with each injection is essential if uniform results are to be obtained. A good guide in the insertion of the needle into the proper layer of the skin is to be able to see its oval opening through the superficial layers of the epidermis. If the fluid has been injected properly, a tense local sharply circumscribed swelling appears, which shows the prominent openings of the hair follicles.

(9) The places of injection on the guinea-pigs are examined at the end of 24, 48, 72, and 96 hours, and a careful record is kept of each test (Table 3). The following changes in the local appearance of the skin at the sites of injection are noted:

- (a) Redness, whether marked (R) or slight (r).
  - (b) Induration, whether marked (Id), moderate (mod), or slight (sl).
  - (c) Necrosis, marked (N) or slight (n), or only superficial scaling (scal).
- The induration is most marked at the end of 48 or 72 hours; the necrosis appears at the end of 72 hours.

The following tables show (1) the amounts of toxin, serum, and salt solution used in the test; (2) the method followed in the examination of several sera; (3) a sample page of the daily record of the tests on the guinea-pig; and (4) an experiment on 4 horses showing the development of antitoxin after injections of small amounts of diphtheria toxin-antitoxin.

TABLE 1  
INTRADERMAL ESTIMATION OF DIPHTHERIA ANTITOXIN

Test Toxin 1 1 c.c. = $\frac{1}{100}$ L+		Normal Salt Solution		Serum		}	=	Undiluted	1:10	1:100	1:1000	1:10,000
0.2	+	0.0	+	0.2	0.01 unit			0.1 unit	1 unit	10 units	100 units	
0.4	+	0.2	+	0.2	0.02 unit			0.2 unit	2 units	20 units	200 units	
1.0	+	0.8	+	0.2	0.05 unit			0.5 unit	5 units	50 units	500 units	
2.0	+	1.8	+	0.2	0.10 unit			1.0 unit	10 units	100 units	1000 units	
Every 0.2 c.c. of toxin solution added is equal to.....								0.01 unit	0.1 unit	1 unit	10 units	100 units

TABLE 2  
ESTIMATION OF DIPHTHERIA ANTITOXIN BY THE INTRADERMAL METHOD

<p>GUINEA-PIG 1</p> <div><div>A</div><div>B</div></div> <div><div><div>1/10</div><div></div></div><div><div></div><div>1/10</div></div></div> <div><div>C</div><div>D</div></div> <div><div></div><div></div></div> <p>A = 1/10+    C = 1/10- B = 1/10+    D = 1/10+</p>	<p>GUINEA-PIG 2</p> <div><div>A</div><div>B</div></div> <div><div>1:10</div><div></div></div> <div><div></div><div>1/20</div></div> <div><div></div><div>1/2</div></div> <div><div></div><div>1/100</div></div> <p>A = 1/2+    B = 1/100-</p>	<p>GUINEA-PIG 3</p> <div><div>C</div><div>D</div></div> <div><div>1:10</div><div></div></div> <div><div></div><div>1/5</div></div> <div><div></div><div>1/2</div></div> <div><div></div><div>1/2</div></div> <p>C = 1/2    D = 1/2+</p>	<p>GUINEA-PIG 4</p> <div><div>A</div><div>D</div></div> <div><div>1:10</div><div></div></div> <div><div></div><div>1</div></div> <div><div></div><div>2</div></div> <div><div></div><div>2</div></div> <p>A = 1    D = 2+</p>
<p>GUINEA-PIG 5</p> <div><div>D</div><div>A</div></div> <div><div>1:10</div><div></div></div> <div><div></div><div>1/2</div></div> <div><div></div><div>10</div></div> <div><div></div><div>1</div></div> <p>D = 10    A = 1</p>	<p>GUINEA-PIG 6</p> <div><div>E</div><div>F</div></div> <div><div>1:10</div><div></div></div> <div><div></div><div>1/100</div></div> <div><div></div><div>1</div></div> <div><div></div><div>1/10</div></div> <p>E = 1+    F = { 1/100+                   1/10- }</p>	<p>GUINEA-PIG 7</p> <div><div>Standard antitoxin</div></div> <div><div>1:10</div><div></div></div> <div><div></div><div>1/2</div></div> <div><div></div><div>1</div></div> <div><div></div><div>2</div></div> <p>1 c.c. = 1 unit</p>	<p>GUINEA-PIG 8</p> <div><div>Standard antitoxin</div></div> <div><div>1:10</div><div></div></div> <div><div></div><div>9/10</div></div> <div><div></div><div>5/10</div></div> <div><div></div><div>1</div></div> <p>1 c.c. = 1 unit</p>

Rectangles indicate blood sera and their dilutions. Circles indicate results of tests, the white ones meaning no lesion, the black ones local necrosis, and the shaded ones slight local inflammatory lesions.

TABLE 3  
SAMPLE PAGE OF DAILY RECORD OF TESTS

Sera	Guinea-pig	Place of Injection	Amount of Antitoxin Tested	Daily Observations				Result of Tests
				Feb. 13	Feb. 14	Feb. 15	Feb. 16	
A	1	R U	1/10 unit	OK	OK	OK	OK	1/10 +
B	1	B L	1/10 unit	Sl r	Mod R	Id n R	Id N R	1/10 -
C	1	L U	1/10 unit	OK	OK	OK	OK	1/10 +
C	1	L L	1/5 unit	Sl r	Mod R	Mod n R	Mod n R	1/5

R U, R L, L U, L L refer to the right upper, right lower, left upper, and left lower quadrants, respectively, of the guinea-pig abdomen.  
OK = no reaction; Sl = slight induration; Mod = moderate induration; Id = marked induration. N = marked necrosis; n = slight necrosis. R = marked redness; r = slight redness.

TABLE 4  
 TESTS ON CONTROL BLEEDINGS TAKEN BEFORE THE INJECTION OF THE TOXIN-  
 ANTITOXIN, AND ON 2 TEST BLEEDINGS TAKEN AFTER THE INJECTION

Horse Injected	Injections of Toxin-Antitoxin "M"	Control Bleeding Dec. 31	Test- Bleeding 1 Jan. 14	Test- Bleeding 2 Jan. 21	Results of Tests		
					Control	Test 1	Test 2
644	1/2 0.25 c.c.	1/27 0.1+	2/2 0.5+	2/10 4+	0.2	4	6
	1/8 0.25 c.c.	1/30 0.01+	2/5 0.1+	2/13 8	0.02	2	8
645	1/2 0.25 c.c.	1/27 0.1-	2/8 0.1+	2/15 0.05+	0.005-	0.05	0.1
	1/2 1 c.c.	1/30 0.01-	2/12 0.05	2/18 0.1			
648	1/2 1 c.c.	1/27 0.1-	2/8 1+	2/15 8	0.5	8	8
	1/2 2 c.c.	1/30 0.2+	2/12 4+	2/18 16-			
651	2/8 2 c.c.	1/30 0.5	2/12 8	2/22 4+			

Guinea-pig 1 (Table 2) shows the preliminary results of tests on 4 different sera for 1/10 unit of antitoxin. Sera A, C, and D completely neutralized the toxin solution and no lesions appeared. Serum B gave a necrosis. Result: A, C, and D had more than 1/10 unit, B less than 1/10 unit of antitoxin to the cubic centimeter.

Guinea-pigs 2 and 3 represent the 2nd set of tests on the 4 sera. Serum A showed no lesion with the 1/2-unit test, therefore more than 1/2 unit was present. Serum B showed less than 1/100 unit, a necrosis appearing with this test. The serum can be further tested for 1/200 unit and if a definite lesion appears the serum is considered as having no antitoxin. Serum C showed no lesion with 1/5 unit, but a slight lesion with 1/2 unit. Antitoxin content therefore was 1/2 unit to the cubic centimeter. If a marked lesion had developed, an intermediate test for 1/3 unit could be made. Serum D had more than 1/2 unit.

Guinea-pig 4 represents the 3rd set of tests for Sera A and D. Serum A showed a lesion with tests for 1 and 2 units. The antitoxin content was therefore 1 unit, since the test in Guinea-pig 2 with Serum A for 1/2 unit showed no lesion. Serum D showed more than 2 units.

Guinea-pig 5 represents the 4th set of tests for Serum D. A lesion appeared with the test for 10 units and no lesion with the one for 5 units. The antitoxin content therefore equaled 10 units. Serum A was retested for 1/2 and 1 unit to show the nonnecrosing and necrosing test points on the same animal.

By using 5 animals we were able to make 20 different tests, and arrive accurately at the antitoxin content of 4 different sera.

Guinea-pig 6 shows 2 sera, E and F, on which the tests were made for widely separated antitoxin contents. This can be done when we wish to arrive rapidly at rough limits of the amount of antitoxin in a serum. Serum E showed more than 1 unit, Serum F more than 1/100, but less than 1/10 unit. Serum E would have to be tested further for 10, 100 units, etc., while Serum F should be tested for 1/20 and 1/50 unit, if we wish to establish the accurate amount of antitoxin.

Guinea-pig 7 shows tests made by this method on the standard antitoxin furnished from the Hygienic Laboratory in Washington. Each cubic centimeter represents exactly one unit of antitoxin. With the intradermal test the antitoxin content was also definitely established as 1 unit to the cubic centimeter, no lesion appeared with the test for 1/2 unit, but a distinct lesion with slight necrosis with the test for

1 unit. The tests for  $1\frac{1}{2}$  and for 2 units showed more marked necrosing lesions.

Guinea-pig 8 shows the same standard antitoxin tested for 0.7, 0.8, 0.9, and 1 unit. Slight inflammatory lesions were noticed even with the test for 0.7 unit. It is therefore best not to attempt to make too fine a differentiation between the slight lesions of closely lying tests, but to endeavor to obtain the necrosing and nonnecrosing points of a serum, the first representing twice the amount of antitoxin tested for in the second. When we are dealing with concentrated antitoxic sera (100-1000 units to the cubic centimeter) the intradermal test can give us only a preliminary idea of the antitoxin content; the more accurate estimation is subsequently made by the subcutaneous test.

#### CONCLUSIONS

An intradermal test is presented for estimating in an economical way small amounts of antitoxin.

The method is easily carried out, tho it requires for accuracy a uniform technic in making the dilutions of toxin and serums, the allowance of a uniform time period for the two to combine, uniformity in the method of injection into the guinea-pig, daily observations of the animals, and uniform interpretation of the tests.

Problems in experimental work in diphtheria and the accurate serologic control of actively immunized individuals can be carried out by this test.

## DIPHTHERIA BACILLI IN MICROSCOPICAL SECTIONS OF TONSILS FROM DIPHTHERIA-CARRIERS \*

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When it was found that certain persistent diphtheria-carriers promptly gave negative cultures after tonsillectomy and removal of adenoids as reported by Friedberg<sup>1</sup> and Ruh, Miller, and Perkins,<sup>2</sup> it was thought interesting to section the tonsils of diphtheria-carriers and study them for the presence and location of diphtheria bacilli. Albert<sup>3</sup> thought from clinical evidence that the diphtheria bacilli multiplied deep in the crypts, and Kretschmer<sup>4</sup> reported success in clearing up diphtheria-carriers by squeezing the tonsils so as to force the plugs of material out of the crypts. Ruh, Miller, and Perkins got positive cultures from the crypts after excision when surface cultures were negative.

The tonsils studied were from 7 diphtheria-carriers, 6 of whom had had pharyngeal diphtheria and 1 of whom was a carrier without symptoms of intoxication. All had had positive cultures from nose or throat for 21 days or over. As controls, tonsils were studied from 14 patients in the nose and throat clinic of the Cook county hospital. These patients had undergone tonsillectomy for various causes such as chronic tonsillitis, hypertrophied tonsils, arthritis, and sciatica. Unfortunately, cultures were not made from 9 of these patients, so that the possibility of their being diphtheria-carriers was not ruled out.

The tonsils were fixed in a solution of equal parts of absolute alcohol and saturated bichlorid of mercury, run through paraffin, cut, and stained by the Gram-Weigert method. Of the 7 diphtheria-carriers 6 yielded gram-positive beaded bacilli in great number. These bacilli, together with large and small streptococci and diplococci, were found chiefly in the crypts in the plugs of tissue, composed of cell debris, lymphoid cells, and polymorphonuclear leukocytes (Figs. 1 and 2).

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<sup>1</sup> Jour. Am. Med. Assn., 1916, 66, p. 816.

<sup>2</sup> Ibid., p. 941.

<sup>3</sup> Ibid., 1913, 61, p. 1027.

<sup>4</sup> Med. Klin., 1911, 7, p. 99.

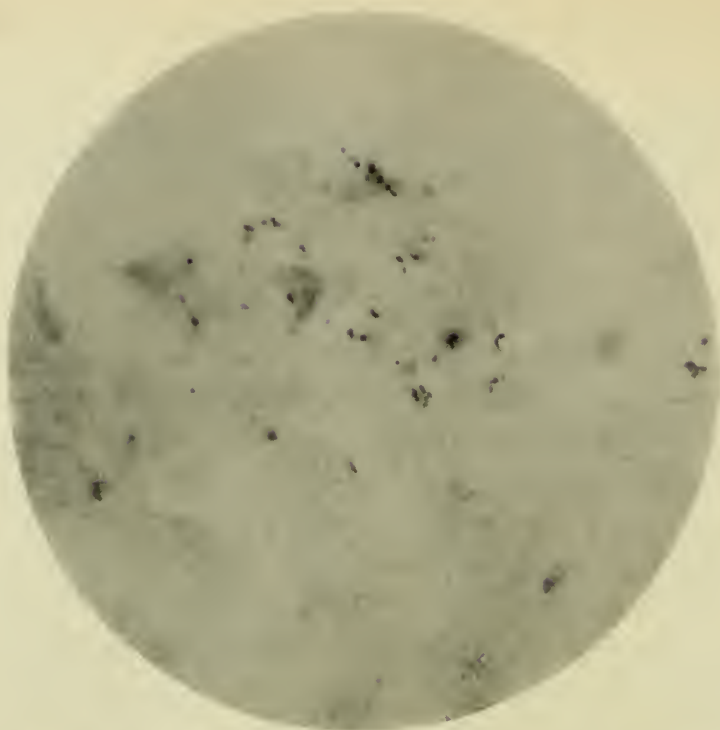


Fig. 1. Plug of tissue in a crypt, the tissue being composed of lymphoid cells and cell debris, showing gram-positive beaded bacilli.

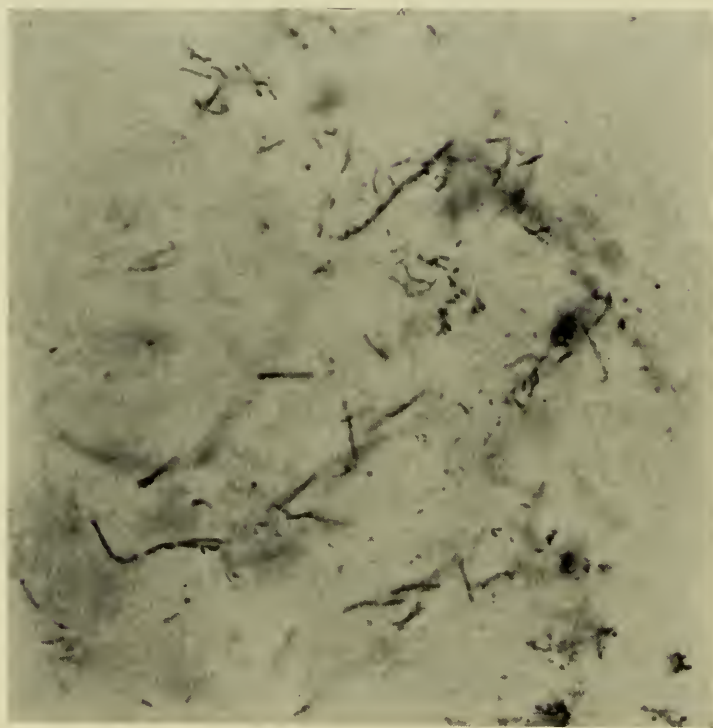


Fig. 2. Base of a crypt where the bacilli are embedded in the tonsillar tissue itself.

The bacilli were found also in the tissues lining the crypts where the epithelium was very thin. There was little or no reaction in these places on the part of the tissues; no new growth of connective tissue or accumulation of phagocytic cells (Fig. 3). The cells in the vicinity of the bacteria stained diffusely and showed some slight karyorrhexis. At some point in each of these 6 pairs of tonsils and in some cases in several places, the epithelium of the surface of the tonsil was necrotic and the bacilli were found in the tissues, showing

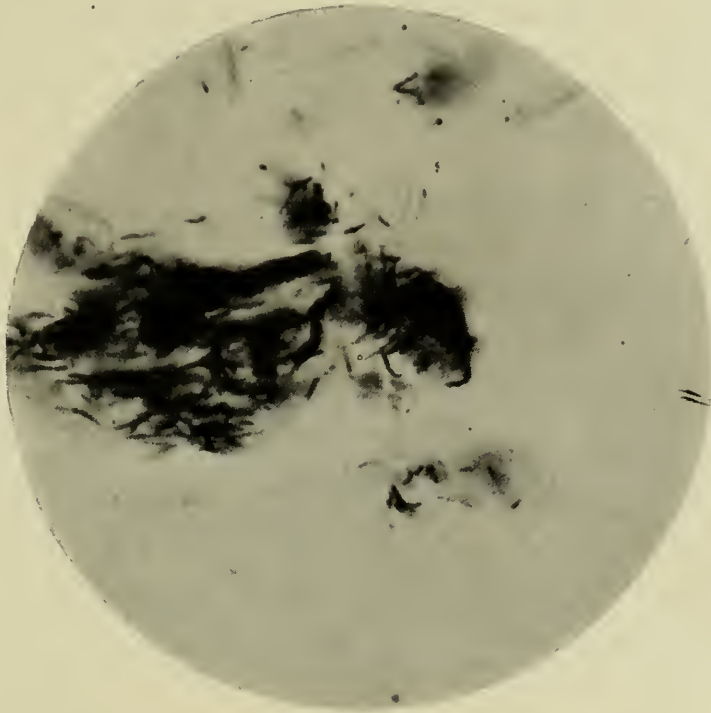


Fig. 3. Side of a crypt showing a depression into which the bacilli are densely packed. The epithelium is intact and the bacilli have not entered the tissues.

that where there are lesions the bacteria multiply on the surface as well as in the crypts. The one case which showed no gram-positive bacilli in 50 sections of the tonsils and in 50 sections of the adenoids, continued to give positive cultures from the throat for 4 days and from the nose for 19 days after tonsillectomy and removal of adenoids. In this case the bacteria were probably multiplying in some place other than the tonsils and the adenoids.

Of the 14 pairs of control tonsils studied only 2 showed any gram-positive bacilli, and these may very well have been diphtheria bacilli. These results correspond with those of Dwyer and Gignoux,<sup>5</sup> who

<sup>5</sup> The Laryngoscope, 1910, 20, p. 1042.

found in a series of 72 cases in which the tonsillar crypts were examined bacteriologically some member of the diphtheria family present in 23%, and other bacilli which are not listed as gram-negative, in 2%. Keitly<sup>6</sup> in a study of the occurrence of diphtheria bacilli in normal throats concludes that the incidence of carriers in adults is from 0 to 2%, and in children from 0 to 25%.

It would seem safe to conclude from these results that the gram-positive beaded bacilli found in the tonsillar crypts of 6 of the diphtheria-carriers were probably diphtheria bacilli and not other gram-positive bacilli, which are relatively rare in the tonsillar crypts, and that the clearing up of these cases by tonsillectomy can be attributed to the removal of an important focus of infection in which the bacilli had lodged and multiplied.

<sup>6</sup> New York Med. Rec., 1915, 88, p. 311.

# THE ATTENUATION OF HOG-CHOLERA VIRUS\*

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Many attempts have been made to modify the virus of hog cholera. The results have thus far been quite unsatisfactory. Graham and Brueckner<sup>1</sup> concluded from their studies that virus attenuated by heat is worthless in rendering swine immune to cholera. Craig<sup>2</sup> reached much the same conclusion. Lewis and others<sup>3</sup> apparently protected 4 pigs with blood drawn from a hyperimmune hog 24 hours after hyperimmunization.

During the summer of 1914, while working on the etiology of hog cholera, at the suggestion of Dr. Kastle we endeavored to modify hog-cholera virus by incubating at 37 C. a mixture of virus blood and hyperimmune blood.

Three cubic centimeters of fresh noncarbolized defibrinated hog-cholera blood were obtained as aseptically as possible and placed in a sterile Erlenmeyer flask; 20 c.c. of hyperimmune defibrinated blood containing 0.5% carbolic acid were added to the virus blood. The hyperimmune blood had been at 10 C. for 6 weeks. The flask was then placed at 37 C. At the end of 24 hours the appearance of the preparation was unchanged. At the end of 48 hours the preparation was of a deep chocolate color, and somewhat thickened in consistency, with some precipitation. At the end of 48 hours at 37 C. the preparation was given to 2 normal hogs weighing about 100 lb. each. The hogs each received 10 c.c. of the preparation by hypodermic injection into the muscles of the right inner ham.

For 2 weeks following the injections the animals remained normal, their temperatures ranging from 101 to 103 F. They were then placed in the exposure pens. The following are the temperature charts for these hogs during the period of their exposure in the cholera pens. On August 1, 1914, these hogs being normal were given to the serum laboratory.

We made a further attempt to modify hog-cholera virus by incubating at 37 C. a mixture of virus blood and the fresh serum of an animal other than the hog.

\* Received for publication June 4, 1916.

<sup>1</sup> Jour. Med. Research, 1915, 31, p. 557.

<sup>2</sup> Ann. Rep. Purdue Univ. Agr. Exper. Sta., No. 28, 1914, p. 73.

<sup>3</sup> Bull. Okla. Agr. Exper. Sta., No. 104, 1914.

HOG 67, JULY, 1914

11	12	13	14	15	16	17	18	20	21	22	23	24	25
103.5 F.	102.8	103.8	104	103.8	103.4	104.6	104.1	101.3	101.8	101.2	101.8	102.1	101.6

HOG 68, JULY, 1914

11	12	13	14	15	16	17	18	20	21	22	23	24	25
100.6 F.	101	101.3	102.2	101.8	102.5	102.2	102.3	101	103	102.5	101.6	101	101.2

Four and five-tenths cubic centimeters of fresh noncarbolized defibrinated hog-cholera blood were obtained as aseptically as possible and placed in a sterile Erlenmeyer flask; 30 c.c. of normal rabbit serum were added to the virus blood. The flask was then placed at 37 C. At the end of 24 hours there was some precipitation together with a slightly increased cloudiness. At the end of 48 hours the preparation was unchanged. It was then given to 3 normal hogs weighing about 100 lb. each. The hogs each received 10 c.c. of the preparation by hypodermic injection into the muscles of the right inner ham.

For 10 days following the injections the animals remained normal; on the 11th day, however, Hogs 70 and 71 did not seem well and would not eat. On the 12th day they were sick and had diarrhea. This sickness continued until they died, Hog 70 dying on the 22nd day following the injection, Hog 71 on the 16th day. Postmortem examination revealed acute hog cholera in each case.

The third hog in this series remained normal for 2 weeks following the injection, the temperature ranging from 100.2 to 103.6 F. At the end of 2 weeks this hog was placed in the exposure pens together with Hogs 67 and 68. The following is the temperature chart for Hog 69. (Hogs 70 and 71 developing a fatal cholera from the original injection, were not placed in the exposure pens.) August 1, 1914, Hog 69 being normal was given to the serum laboratory.

HOG 69, JULY, 1914

11	12	13	14	15	16	17	18	20	21	22	23	24	25
102.2 F.	102.1	101.8	102.3	101	101.5	101.4	102.1	100	100.6	101	101	101.3	101.3

It is of course impossible to draw positive conclusions from such a small number of experiments, yet we may conclude that, in so far as these experiments go, the virus of hog cholera on incubation with hyperimmune blood for 48 hours at 37 C. is so modified that when injected it will no longer render normal hogs sick, but will protect them when later they are exposed to cholera. We may further conclude that the virus of hog cholera on incubation with normal rabbit serum for 48 hours at 37 C. is modified to the extent that 1 of 3 animals was protected against cholera.

It is quite possible that the mixture of hog-cholera virus and hyperimmune blood which had been modified by incubation for 48 hours at 37 C. and then rapidly dried over sulfuric acid at a low temperature, would indefinitely retain its power to protect hogs against cholera, and yet not sicken them.

# THE SPECIFICITY OF STREPTOCOCCI\*

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## INTRODUCTION

The differentiation of streptococci is one of the most perplexing problems of bacteriology. As early as 1884, Rosenbach<sup>1</sup> and Fehleisen<sup>2</sup> claimed for the streptococci which they had isolated, specificity for abscesses and erysipelas respectively, but Petruschky<sup>3</sup> was able to show that the same strain might produce either condition. The more recent work on the specificity of streptococci has developed along several distinct lines.

The carbohydrate-fermentation reactions, which have proved so successful in the differentiation of members of the colon-typhoid group, were first applied to streptococci by Gordon<sup>4</sup> and Houston,<sup>5</sup> whose work was amplified by Andrewes and Horder<sup>6</sup> and in America by Winslow<sup>7</sup> and others.<sup>8</sup> The value of these tests was questioned by Walker,<sup>9</sup> Buerger,<sup>10</sup> Bergey,<sup>11</sup> and Thro,<sup>12</sup> who found the reactions variable; but a general review of the literature indicates that the majority of observers have found the fermentation tests constant enough to warrant a division of the streptococcus group into a number of distinct varieties.

The classification of Schottmüller<sup>13</sup> based on the characters of the colonies on blood-agar plates has been more generally accepted. But even the constancy of the hemolytic property was denied by Ruediger,<sup>14</sup> Anthony,<sup>15</sup> and especially by Rosenow,<sup>16</sup> who found all the members of this group, including the encap-

\* Received for publication May 18, 1916. This work was aided by a grant from the graduate school of the University of Minnesota.

<sup>1</sup> Mikroorganismen bei Wundkrankheiten, 1884.

<sup>2</sup> Aetiologie der Erysipelas, 1883.

<sup>3</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1896, 23, p. 142.

<sup>4</sup> Rep. Local Govt. Board, London, 1903, 33, p. 85. Jour. Path. and Bacteriol., 1911, 15, p. 323.

<sup>5</sup> Rep. Local Govt. Board, London, 1903-4, 33, p. 472; 1904-5, 34, p. 326. Rep. to London County Council on Milk, 1905.

<sup>6</sup> Lancet, 1906, 2, p. 708.

<sup>7</sup> Jour. Infect. Dis., 1912, 10, p. 285.

<sup>8</sup> Winslow and Palmer: Jour. Infect. Dis., 1910, 7, p. 1. Broadhurst: Ibid., 1912, 10, p. 272; 1913, 13, p. 404; 1915, 17, p. 277. Stowell, Hilliard, and Schlesinger: Ibid., 1913, 12, p. 144. Fuller and Armstrong: Ibid., 13, p. 442. Hopkins and Lang: Ibid., 1914, 15, p. 163. Floyd and Wolbach: Jour. Med. Research, 1914, 29, p. 493. Lyall: Ibid., 30, p. 487.

<sup>9</sup> Proc. Roy. Soc. London, 1911, 83, Series B, p. 541.

<sup>10</sup> Jour. Exper. Med., 1907, 9, p. 428.

<sup>11</sup> Jour. Med. Research, 1912, 27, p. 67.

<sup>12</sup> Jour. Infect. Dis., 1914, 15, p. 234; 1915, 17, p. 227.

<sup>13</sup> München. med. Wchnschr., 1903, 1, p. 849; 1910, 57, p. 617.

<sup>14</sup> Jour. Infect. Dis., 1906, 3, p. 663.

<sup>15</sup> Ibid., 1909, 6, p. 332.

<sup>16</sup> Ibid., 1914, 14, p. 1.

sulated forms, readily transmutable. It is to be noted, however, that Holman<sup>17</sup> failed to confirm the results of Rosenow's experiments and pointed out possible sources of error.

Several attempts have been made to throw light on this problem by immunologic studies. Floyd and Wolbach<sup>18</sup> found that agglutination and complement-fixation tests supported the evidence yielded by carbohydrate fermentations. Kligler<sup>19</sup> arrived at similar conclusions from his studies of agglutinins; he found a closer affiliation from an immunologic standpoint between strains fermenting the same carbohydrates than between strains having similar hemolytic properties. Davis,<sup>20</sup> on the other hand, from a study of anaphylaxis in cross-sensitization experiments, found a very close relationship between the hemolytic strains, and in this relationship the nonhemolytic strains did not participate.

Aside from these biochemical studies there has accumulated a considerable amount of literature indicating the occurrence of specific differences between various streptococci in respect to virulence and the localization and characters of the lesions produced. This work had its beginnings in the observations of Poynton and Paine<sup>21</sup> and reached a climax in the recent publications of Rosenow.<sup>22</sup> It is not necessary to review this literature here; references will be made to portions of it in the discussion of my own experiments.

There is very little literature describing experimental work tending to correlate these various series of observations. Andrewes and Horder characterized their *S. pyogenes* and *S. anginosus* as pathogenic and their *S. salivarius* and *S. faecalis* as nonpathogenic. Later Horder<sup>23</sup> noted that *S. salivarius* and *S. faecalis* were frequently associated with malignant endocarditis. Beattie and Yates<sup>24</sup> compared the carbohydrate fermentations with virulence for rabbits; they experienced difficulty in classifying their streptococci, having a large group of variants. Three strains of *S. pyogenes* produced septicemia; 20 strains of *S. salivarius* caused septicemia 6 times, arthritis 10 times, endocarditis once, and no results in 5 rabbits; 6 strains of *S. faecalis* produced septicemia once and arthritis 5 times; 3 strains of *S. anginosus* caused septicemia once and produced no results in 2 rabbits. They concluded that the sugar tests, from the standpoint of sources and pathogenic effects of streptococci, are of no practical value. Floyd and Wolbach<sup>18</sup> noted an inverse relationship between virulence and fermentative powers. Heinemann,<sup>25</sup> also, observed that repeated animal passage, tho raising virulence, decreased the fermentative powers.

Schottmüller found his *S. longus-seu-erysipelatos* associated particularly with phlegmons, erysipelas, and septicemia, and his *S. viridans-seu-mitior* present in localized or subacute infections, especially endocarditis; these findings have been generally confirmed by clinical observations since. Rosenow<sup>26</sup> demonstrated by experimental methods a very close relationship between certain strains of *S. viridans* (which he considers to be mutants of *Pneumococcus*) and endocarditis.

<sup>17</sup> Ibid., 15, p. 293.

<sup>18</sup> Jour. Med. Research, 1914, 29, p. 493.

<sup>19</sup> Jour. Infect. Dis., 1915, 16, p. 327.

<sup>20</sup> Ibid., 1913, 12, p. 386.

<sup>21</sup> Researches on Rheumatism, 1914.

<sup>22</sup> Jour. Am. Med. Assn., 1915, 65, p. 1687.

<sup>23</sup> Quart. Jour. Med., 1908-9, 2, p. 289.

<sup>24</sup> Jour. Path. and Bacteriol., 1911, 16, p. 247.

<sup>25</sup> Jour. Infect. Dis., 1915, 16, p. 221.

<sup>26</sup> Ibid., 1910, 7, p. 411; 1912, 11, p. 210.

Davis<sup>20</sup> published a table indicating that hemolytic streptococci, when inoculated into rabbits, have a pronounced affinity for the joints not shown by the viridans strains, the latter exhibiting a greater affinity for the heart valves.

M'Leod<sup>27</sup> made an exhaustive review of the literature on the relation of hemolysis to virulence in streptococci. Of 16 authors who compared hemolysis with virulence by experimental inoculations into laboratory animals, 4 concluded that a relationship could be established, 10 decided it could not, and 2 drew no conclusions. M'Leod criticized the blood-agar-plate method for determining hemolysis, and stated that if the streptococci to be tested be first grown in the serum of the animal used to determine virulence, and hemolysis be determined by titration with salt suspensions of the red cells of that animal, a close relationship may be established between the hemolytic titer and the minimal lethal dose for that animal. (In my work, hemolysis was determined in every instance on rabbit-blood agar, and rabbits alone were used for inoculation.) Lyall,<sup>28</sup> studying hemolysis by titration with sheep cells, concluded that the hemolytic titer was no absolute criterion of virulence.

It is unfortunate that those bacteriologists who have conducted the greater number of animal experiments with streptococci have paid so little attention to these various biochemical characters. Poynton and Paine, in particular, gave surprisingly meagre descriptions of their *Diplococcus rheumaticus*; but Major<sup>29</sup> identified their organisms with the *S. viridans* of Schottmüller, and both as strains of *S. salivarius* or *S. faecalis* of Andrewes and Horder.

The purpose of the work here reported has been to demonstrate by experimental means what relationship, if any, exists between the various biochemical characters of streptococci (hemolytic power and carbohydrate-fermentation reactions) on the one hand, and virulence, elective organ affinities, and the character of the exudate produced, on the other.

#### MATERIAL AND METHODS

This study was limited to the nonencapsulated forms of streptococci found on the body surfaces or in pathologic conditions in man. These were isolated by plating on agar, and as soon as vigorous pure cultures were obtained, they were planted simultaneously into the various carbohydrate media and into flasks of media to be used for inoculating rabbits. The carbohydrate media, lactose, saccharose, mannite, raffinose, salicin, and inulin, were prepared according to the method of Holman,<sup>30</sup> using Andrade's indicator. Hemolysis was determined by plating on rabbit-blood agar (2 drops to the cubic centimeter), usually from the broth cultures used to inoculate the rabbits, thus being insured the purity of the cultures injected. The time of cultivation of the streptococci on artificial media before their inoculation into rabbits averaged 7 days.

Rabbits were injected with the growth from 24-hour cultures in double-strength 2%-dextrose broth to which had been added one-fourth volume of sterile beef serum or human ascitic fluid. As far as possible, young rabbits weighing close to 1,000 gm. were used, but the size varied considerably. Inoculations were

<sup>27</sup> Jour. Path. and Bacteriol., 1915, 19, p. 392.

<sup>28</sup> Jour. Med. Research, 1914, 30, p. 515.

<sup>29</sup> Bull. Johns Hopkins Hosp., 1912, 23, p. 326.

<sup>30</sup> Jour. Infect. Dis., 1914, 14, p. 209.

made in multiples, usually 4 rabbits being injected with the same strain in varying doses. When doses of more than 10 c.c. were given, the centrifugated bacteria, free from broth, were used. In the earlier part of the work, massive doses (as high as 75 c.c.) were injected, as recommended by Rosenow. It was found, however, that many of the rabbits receiving these large doses died in a very short time, apparently from toxemia; and where less virulent cultures were injected, the rabbits receiving very large doses were not much more likely to develop lesions than those inoculated with moderate doses, so that the massive doses were abandoned. Beattie<sup>31</sup> has found that with rheumatic strains the dose is of little importance as far as the development of arthritis is concerned. It was thought desirable, however, to vary the dose somewhat in order to obtain a rough titer of virulence. The average dose was 5 c.c. All injections were made intravenously.

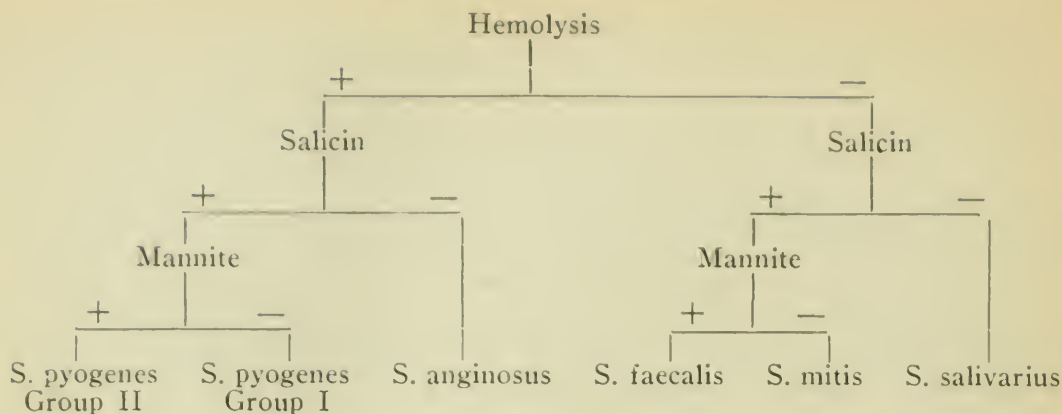
If the animals did not die spontaneously, they were chloroformed at intervals of from 2 to 10 days, as previous experience had shown that many of the lesions produced are more or less evanescent, a majority of the rabbits tending to recover completely from their infections. Except for a few instances, complete autopsies were made. In the case of the first 100 rabbits, routine microscopical examination was made of the arch of the aorta, the heart muscle, spleen, pancreas, liver, gallbladder, appendix, kidneys, and one or more of the lymph nodes, as well as of all other tissues showing gross lesions. I learned from these experiences that lesions were seldom found microscopically which did not appear grossly, so that in subsequent work, I made microscopical examinations only in those rabbits showing some gross evidence of infection, still examining, however, sections of all the organs named. I did not make routine bacteriologic examinations of these rabbits; they were made frequently enough, however, to indicate that the streptococci were present in most of the lesions described. The organisms recovered invariably showed the same fermentative and hemolytic characters as those inoculated.

#### SOURCES AND CLASSIFICATION OF STRAINS

Cultures were derived from many sources. An effort was made to obtain strains of each variety, both those existing as saprophytes on the body surfaces and those exhibiting varying degrees of pathogenicity within the tissues. There is some disproportion in number among the strains of each variety. Thus, as a consequence of the fact that during the course of this work I was also investigating chronic mouth infections, a large number of strains of *S. mitis* from that source are included. I experienced difficulty in finding pathogenic strains of *S. faecalis* and *S. anginosus*. The source of each strain is indicated in tables to follow.

In classifying the strains I adopted the terminology of Andrewes and Horder. A classification based on the fermentation reactions alone presents considerable difficulties; thus, the only criteria offered for the differentiation of *S. mitis* and *S. pyogenes* are the length of the chains and the pathogenicity for mice, both of which are probably highly variable characters. If the sugar tests be considered in relation to the presence or absence of hemolysis, however, the classification becomes relatively simple. The terminology used is indicated in the following diagram:

<sup>31</sup> Jour. Path. and Bacteriol., 1910, 14, p. 432.



As will be seen, it was found desirable to divide *S. pyogenes* into 2 groups. Group II differs from the type species in fermenting mannite. By the sugar tests alone these would be classified as *S. faecalis*, and Lyall<sup>28</sup> suggested that these strains represent varieties of *S. faecalis* which have acquired hemolytic properties,, but there seems to be no clear evidence for this. Practically all strains fermented lactose and saccharose and acidified litmus milk. Only 2 strains fermented inulin; they failed to produce capsules in mice and were classified as variants of *S. salivarius* and *S. mitis* respectively. Raffinose, according to Andrewes and Horder's tables, is fermented regularly only by *Pneumococcus*. In our series it was usually fermented by the strains classified as *S. salivarius*, and by most strains of *S. mitis*, but seemed to serve no useful purpose in the classification and was therefore not considered.

#### TABULATION OF RESULTS

In Tables 1 to 6 the essential details of the various experiments are presented in condensed form. In the column marked "Died or Chloroformed" the sign + indicates that the animals died spontaneously within the time limits of the experiments. In the columns indicating the distribution of the various lesions, the sign + indicates an infiltrative lesion, and H indicates an interstitial hemorrhage.

#### DESCRIPTION AND DISCUSSION OF LESIONS

The lesions produced may be divided into 2 groups, hemorrhagic and infiltrative. The hemorrhagic lesions (the importance of which has been emphasized by Rosenow) occurred in the capsular tissues of the joints, in the voluntary muscles, heart valves, and occasionally in the heart muscle and in the kidneys. In several instances hemorrhages also were noted in the stomach, in the small intestine, and in the appendix. For the most part these hemorrhages were small, but occasionally they were extensive, involving for instance all the muscles of one extremity. Microscopically, these lesions usually showed nothing but interstitial hemorrhages; occasionally, however, the hemorrhagic

areas contained small foci of leukocytic infiltration, so that it seems reasonable to suppose that the hemorrhagic condition precedes the infiltrative lesion. No attention was paid to parenchymatous changes, such as fatty infiltration in the heart, cloudy swelling of the kidneys, etc.

*Nervous System.*—The central nervous system was not examined routinely in these rabbits, but some of them presented certain nervous symptoms and lesions in the brain which are interesting when considered in connection with the published reports on the relation of streptococci to chorea.

Dana<sup>32</sup> observed diplococci in sections of the brain in chorea, and Apert<sup>33</sup> cultivated a diplococcus from the blood. Wassermann, Westphal, and Malkoff<sup>34</sup> isolated a diplostreptococcus from the blood and from the brain in a fatal case of chorea; this organism produced arthritis in rabbits. Poynton and Paine<sup>21</sup> repeatedly cultivated their "diplococcus" from the blood and spinal fluid in cases of chorea and were able to demonstrate the organism in the pia matter and in the vessel walls of the brain. They also noted the occurrence of choreic symptoms in one of a series of rabbits inoculated with streptococci from rheumatism, and found the organism in the rabbit's brain. Beattie,<sup>35</sup> too, observed choreic movements in an inoculated rabbit, and found an exudate of polymorphonuclear cells on the surface of the cord in places. Cole<sup>36</sup> noted the occurrence of convulsive incoordinate movements in 2 rabbits injected with streptococci from cases of septicemia, but did not believe that the symptoms resembled those of chorea.

The pathologic changes occurring in rheumatic chorea are not well known. Poynton and Holmes<sup>37</sup> described vascular changes, hyperemia, multiple thromboses with softening, and, more constantly, perivascular small-round-cell infiltration in the pia and in the brain substance; also degenerative changes in the nerve cells and fibers.

The symptoms shown by our rabbits, which were probably identical with those described by the investigators quoted, may be best characterized as an inability to keep still. There were constant movements of the head from side to side, and in walking there was marked staggering, with a very apparent inability to coordinate movements. Retraction of the neck was noted in one animal.

Grossly the brains appeared normal, but microscopically there were found in all 4 rabbits lesions which varied only in degree. In the pia there were congestion and occasional microscopic hemorrhages, with a cellular infiltration that varied from a few perivascular lymphocytes in one case to a diffuse infiltration of the entire brain surface in others. This layer of inflammatory cells was thin, and altho it showed numerous pus cells in many places, mononuclear cells predominated. Within the brain substance there were areas of peri-

<sup>32</sup> Am. Jour. Med. Sc., 1894, 108, p. 31.

<sup>33</sup> Compt. rend. Soc. de biol., 1898, 5, p. 128.

<sup>34</sup> Berl. klin. Wehnschr., 1899, 36, p. 638.

<sup>35</sup> Jour. Path. and Bacteriol., 1904, 9, p. 272.

<sup>36</sup> Jour. Infect. Dis., 1904, 1, p. 714.

<sup>37</sup> Lancet, 1906, 2, p. 982.

TABLE 1  
STREPTOCOCCUS PYOGENES, GROUP I

Strain	Source	Days Cultivated	Rabbit	Dose in c.c.	Duration of Infection, Days	Died or Chloroformed*
6	Nose.....	4	30	5	5	0
			31	15	5	0
			32	45	5	0
33	Sputum.....	6	107	45	1	+
			108	15	1	+
			109	5	2	+
			110	5	2	+
44	Dental fistula.....	5	152	10	0	+
			153	5	5	0
			154	5	5	+
			155	5	5	0
48	Tonsil.....	17	156	6	1	+
			157	2	1	+
			159	8	1	+
			166	4	2	+
65	Blood culture (septicemia of obscure origin)	7	168	10	8	0
			169	5	8	0
			170	8	8	0
96	Empyema.....	1	205	5	1	+
			206	5	1	+
			207	5	1	+
			208	5	1	+
			209	1	3	+
			210	1	3	+
			211	1	3	+
119	Blood culture (septicemia).....	4	217	5	4	0
			218	3	4	0
			219	1	4	0
			220	1	4	0
			221	1/2	4	0
112	Blood culture (septicemia).....	1	226	5	5	0
			227	5	5	0
			228	3	4	0
			229	1	5	0
114	Knee-joint (arthritis following pneumonia)	1	234	8	1	+
			235	5	6	+
			236	5	8	0
			237	1	8	0
122	Peritonitis.....	1	238	10	1	+
			239	5	2	+
			240	3	2	+
			241	1	7	0
123	Acute endocarditis following erysipelas	1	242	10	4	+
			243	5	6	0
			244	5	6	0
			245	1	6	0

\* In this column the sign + indicates that the animal died spontaneously within the time limits of the experiment. In the columns showing the distribution of the various lesions, the sign + indicates an infiltrative lesion, and H indicates an interstitial hemorrhage. This key applies to all of Tables 1 to 6.

TABLE 1—Continued

STREPTOCOCCUS PYOGENES, GROUP I

Heart Muscle	Heart Valves	Joints	Voluntary Muscles	Lymph Nodes	Spleen	Kidneys	Remarks
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	+	0	0	
0	0	0	0	+	0	0	
0	0	0	0	+	+	0	
0	0	H	0	0	+	+	Subacute meningitis ("chorea")
0	0	0	0	0	0	0	Died in a few hours
+	+	+	0	0	0	+	
+	+	0	0	0	0	+	
0	0	0	0	0	0	0	
0	0	0	0	0	+	0	
+	0	0	0	0	+	0	
0	0	0	0	0	+	0	Hemorrhages in appendix
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	
0	C	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	C	0	0	0	0	0	
+	0	+	+	0	0	0	
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	C	0	0	0	0	
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	+	0	0	0	+	Fibrinous pericarditis
0	0	+	0	0	0	0	
+	H	0	H	0	0	0	
+	+	0	0	+	+	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
+	+	0	0	0	0	0	
+	0	0	0	0	0	0	
0	0	0	0	0	0	+	Sero-fibrinous pericarditis

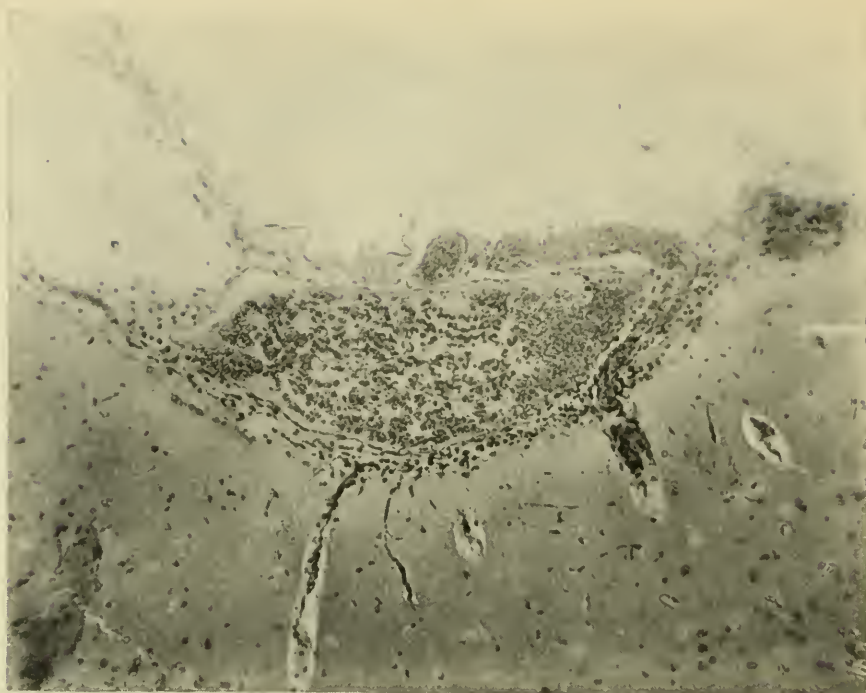


Fig. 1. "Chorea." Perivascular round-cell infiltration in the pia mater.

vascular lymphocytic infiltration, and minute foci of mononuclear cells not associated with vessels. Beneath the ependyma of the ventricles there was a diffuse lymphocytic infiltration. These changes were not confined to the cerebrum but were present in the cerebellum and pons as well. The condition may be considered a subacute encephal meningitis.

It is not certain that either the symptoms or the lesions in these rabbits have anything in common with rheumatic chorea in man. In any event, the condition cannot be considered specific, since it was produced once by *S. pyogenes*, once by *S. mitis*, and twice by *S. salivarius*.

*Heart Valves.*—The infectious nature of endocarditis has long been known, and a variety of organisms have been associated with this disease; of these, streptococci have been by far the most frequent.

The more or less specific characters of these streptococci were established by the studies of Schottmüller,<sup>13</sup> Horder,<sup>23</sup> and Libman and Cellar.<sup>33</sup> Poynton and Paine<sup>21</sup> also cultivated streptococci from cases of endocarditis which they considered identical with those obtained from rheumatism; further, they obtained the same organism from cases of malignant endocarditis and were able to produce this type of disease in rabbits with streptococci from cases of simple rheumatism. They concluded that their work had established firmly the relationship between rheumatism and both simple and malignant endo-

<sup>33</sup> Am. Jour. Med. Sc., 1910, 140, p. 516.

carditis. In this connection it is interesting to note that Thalheimer and Rothschild<sup>30</sup> failed to find the specific lesions of rheumatic myocarditis in cases of endocarditis due to *S. viridans*.

Endocarditis has been produced experimentally in animals by a number of observers. Wyssokowitsch<sup>40</sup> found that intravenous injections of staphylococci and streptococci into rabbits failed to produce endocarditis unless the valves had previously been injured by operative manipulation (this manipulation, as had been shown by Rosenbach,<sup>41</sup> did not in itself produce inflammatory changes in the valves of rabbits) and Orth<sup>42</sup> interpreted these experiments as indicating that the disease required for its development not only the presence of circulating micro-organisms, but also a predisposing factor in the valves themselves. The experiments of Wyssokowitsch were repeated and confirmed by Fraenkel and Sanger<sup>43</sup> and by Weichselbaum.<sup>44</sup> Prudden<sup>45</sup> accomplished the same end by previously treating the valves with caustics. Ribbert<sup>46</sup> produced endocarditis by injecting staphylococci simultaneously with an emulsion of potato, again producing a predisposing condition, according to Orth's interpretation, either in the adherence of the potato particles to the valves and chordae or in the production of emboli. Bonome,<sup>47</sup> however, observed the development of endocarditis in rabbits merely injected with cultures of staphylococci, if these formed clumps large enough to constitute emboli. Successful results with staphylococci were also obtained by Josserand and Roux<sup>48</sup> and by Lissauer.<sup>49</sup>

Dreschfeld,<sup>50</sup> apparently, was the first to succeed in producing endocarditis by the simple inoculation of streptococci into rabbits. More recently, such observations have been repeatedly made by Poynton and Paine, Beattie,<sup>51</sup> Shaw,<sup>51</sup> and Libman and Cellar.<sup>33</sup> Rosenow has been especially successful with these experiments. Thus, 84% of his rabbits inoculated with streptococci from endocarditis have shown that lesion at autopsy, as compared with 14% of rabbits inoculated with strains from other sources.<sup>22</sup> The apparent affinity of these streptococci (which he considers to be modified pneumococci) for the heart valves, Rosenow believes is related to their ability to produce clumps in liquid, and adherent colonies on solid media; for he finds that endocarditis in rabbits is primarily an embolic process, the organisms lodging within capillaries in the valves and producing first a hemorrhage, later inflammatory infiltration, ending in ulceration and the development of vegetations.<sup>26</sup>

In discussing the heart-valve lesions in our rabbits, then, two questions present themselves for consideration: (1) To what extent do streptococci of various kinds exhibit an affinity for the heart valves,

<sup>30</sup> Jour. Exper. Med., 1914, 19, p. 417.

<sup>40</sup> Virchow's Arch. f. path. Anat., 1886, 103, p. 301.

<sup>41</sup> Arch. f. exper. Path. u. Pharmakol., 1878, 9, p. 1.

<sup>42</sup> Virchow's Arch. f. path. Anat., 1886, 103, p. 333.

<sup>43</sup> Ibid., 1887, 108, p. 286.

<sup>44</sup> Centralbl. f. Bakteriol., 1887, 2, p. 209.

<sup>45</sup> Am. Jour. Med. Sc., 1887, 93, p. 55.

<sup>46</sup> Fortschr. d. Med., 1886, 4, p. 1.

<sup>47</sup> Arch. ital. de biol., 1887, 8, p. 10.

<sup>48</sup> Arch. de med. exper. et d'anat. path., 1892, 4, p. 469.

<sup>49</sup> Centralbl. f. allg. Path. u. path. Anat., 1912, 23, p. 243.

<sup>50</sup> Brit. Med. Jour., 1887, 2, p. 887.

<sup>51</sup> Jour. Path. and Bacteriol., 1904, 9, p. 158.



Fig. 2. Embolic endocarditis. Abscesses have developed within the valve leaflet, surrounded by an intact endothelial surface.

and (2) does the process begin primarily as an embolism within the valve, or as an implantation on the endothelial surface itself?

The valve lesions obtained were either subendothelial hemorrhages (which would not disappear after vigorous washing) or vegetations. Microscopical examination was not made of all the vegetations, several of the hearts being saved as gross specimens. In one case, at least, what appeared grossly as vegetations proved microscopically to be abscesses within the valve, which had developed from the base of the valve, spreading towards its free border and pushing the surfaces apart, so that they were completely surrounded by an intact endothelial surface; here undoubtedly the process developed by embolism but did not begin primarily in the valve itself. In all the other instances, however, there was found on the surface of the valve an exudate of fibrin with leukocytes and masses of cocci, the valve itself showing but little change, certainly no evidences that the process had begun within and later ulcerated through (Fig. 3). We must conclude then that streptococcal endocarditis usually develops by implantation on the surface of the valve.

Our experiments have not revealed specific differences in the affinities of the various classes of streptococci for the heart valves. *S. mitis* produced hemorrhages 4 times and vegetations 3 times; *S. salivarius*, hemorrhage once and vegetations 4 times; *S. faecalis*, vegeta-



Fig. 3. Endocarditis. Vegetation attached to the tip of the valve cusp, the valve structure itself being intact.

tions once. *S. pyogenes* of Group I, caused hemorrhages twice and vegetations 3 times; of Group II, hemorrhage once and vegetations 3 times; *S. anginosus*, vegetations once. Lesions of the heart valves, then, developed in about 9% of the rabbits inoculated with nonhemolytic strains, and in 11% of those which received hemolytic streptococci.

*Heart Muscle.*—Interest in the myocardial lesions produced by streptococci depends on the fact that there occurs in rheumatic fever a type of myocarditis which is apparently specific for that disease.

This lesion has been described by Aschoff,<sup>52</sup> Geipel,<sup>53</sup> Fraenkel,<sup>54</sup> Coombs,<sup>55</sup> Thalheimer and Rothschild,<sup>39</sup> and others. The reaction occurs in circumscribed areas, called submiliary nodules or Aschoff-Geipel bodies. They are found in the intermuscular septa, especially about the vessels. They are composed of large oval or spindle-shaped cells arranged about the vessels in rosettes, or between the muscle fibers in fusiform areas. The nodules are more frequent, according to Coombs, near the endocardium, especially at the bases of the valves. The cells forming these nodules, as shown by Fraenkel, stain red with pyronin-methyl green. Multinucleated giant cells also occur. Coombs found similar lesions in the articular tissues and subcutaneous nodules in cases of rheumatism.

<sup>52</sup> Verhandl. d. deutsch. path. Gesellsch., 1904, 8, p. 46.

<sup>53</sup> Deutsch. Arch. f. klin. Med., 1905, 85, p. 75.

<sup>54</sup> Ziegler's Beiträge, 1912, 52, p. 597.

<sup>55</sup> Jour. path. and Bacteriol., 1910, 15, p. 489. Quart. Jour. Med., 1908-9, 2, p. 26.

TABLE 2  
STREPTOCOCCUS PYOGENES, GROUP II

Strain	Source	Days Cultivated	Rabbit	Dose in c.c.	Duration of Infection, Days	Died or Chloroformed*
32	Sputum (pulmonary abscess)....	6	103	45	5	+
			104	3	5	0
			105	5	5	0
			106	5	5	0
45	Ascites fluid.....	5	144	10	0	+
			145	5	4	+
			146	5	5	0
			147	5	3	+
38	Empyema.....	6	148	10	3	+
			149	5	3	+
			150	5	5	+
			151	5	3	+
111	Empyema (traumatic).....	2	222	5	4	0
			223	3	5	0
			224	1	4	0
			225	1	5	0
113	Meningitis (secondary to decubitus ulcers)	1	230	3	5	0
			231	5	4	0
			232	5	4	0
			233	1	5	0

\* See Table 1 for the significance of + and H.

TABLE 3  
STREPTOCOCCUS ANGINOSUS

Strain	Source	Days Cultivated	Rabbit	Dose in c.c.	Duration of Infection, Days	Died or Chloroformed*
109	Blood culture (septicemia of obscure origin)	4	212	3	4	0
			213	1	4	0
			214	5	4	0
			215	1	4	0
			216	1	4	0
124	Throat (scarlet fever).....	3	246	10	2	+
			247	5	2	+
			248	5	6	0
			249	1	2	+
125	Throat (scarlet fever).....	3	250	10	5	+
			251	5	2	+
			252	5	1	+
			253	1	2	+
126	Throat (scarlet fever).....	3	254	10	1	+
			255	5	4	0
			256	5	1	+
			257	1	4	0
127	Throat (scarlet fever).....	3	258	10	4	0
			259	5	4	0
			260	5	4	0
			261	1	4	0
128	Throat (simple angina).....	2	262	10	1	+
			263	5	3	0
			264	5	1	+
			265	1	3	0
			266	10	2	0

\* See Table 1 for the significance of + and H

TABLE 2—Continued

STREPTOCOCCUS PYOGENES, GROUP II

Heart Muscle	Heart Valves	Joints	Voluntary Muscles	Lymph Nodes	Spleen	Kidneys	Remarks
0	0	0	0	0	0	+	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
+	0	0	0	0	0	+	
0	0	0	0	0	0	0	Died in an hour
+	0	+	0	0	0	+	
0	0	0	0	0	0	+	
+	+	0	0	0	+	+	Multiple septic infarcts of spleen Acute peritonitis
+	0	0	0	0	+	+	
+	+	0	0	0	+	+	
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	+	0	0	0	0	
+	H	+	0	0	0	0	
0	0	0	0	0	0	0	
+	0	+	0	0	0	0	
0	0	+	+	0	0	+	
+	0	+	+	0	0	+	
0	+	+	0	0	0	0	
0	0	0	0	0	0	0	

TABLE 3—Continued

STREPTOCOCCUS ANGINOSUS

[illegible]

TABLE 4  
STREPTOCOCCUS MITIS

Strain	Source	Days Cultivated	Rabbit	Dose in c.c.	Duration of Infection, Days	Died or Chloroformed*
1	Tonsil.....	21	1	1	2	0
			2	5	2	0
			3	5	2	0
			4	5	3	0
			5	5	4	0
			6	5	6	0
			7	5	7	0
			8	15	2	0
			9	45	2	0
2	Saliva.....	3	14	1	2	0
			15	5	2	0
			16	45	2	0
			17	15	2	0
4	Blood culture (subacute endocarditis)	2	22	1	2	0
			23	5	2	0
			24	15	2	0
			25	45	2	0
7	Saliva.....	8	33	1	4	0
			34	5	2	+
			35	15	4	0
			36	45	0	+
8	Saliva.....	8	37	3	6	0
			38	5	6	0
			39	15	6	0
			40	45	0	0
13	Dental abscess.....	21	65	5	6	0
			66	5	6	0
			67	5	6	0
			68	5	6	0
16	Dental abscess.....	4	61	5	4	0
			62	5	1	+
			63	5	4	0
			64	5	10	+
20	Dental abscess .....	4	57	5	4	0
			58	5	4	0
			59	5	4	0
			60	5	4	0
29	Dental abscess.....	7	91	5	4	0
			92	10	4	0
			93	8	4	0
			94	8	4	0
30	Dental abscess.....	7	95	10	4	0
			96	5	3	+
			97	7	4	0
			98	5	4	0
31	Dental abscess.....	6	99	5	4	0
			100	10	4	0
			101	8	4	0
			102	8	4	0
34	Sputum.....	6	111	45	5	0
			112	5	3	+
			113	5	5	0
			114	5	5	0

TABLE 4—Continued  
STREPTOCOCCUS MITIS

Heart Muscle	Heart Valves	Joints	Volun- tary Muscles	Lymph Nodes	Spleen	Kidneys	Remarks
0	0	+	0	0	0	0	
0	0	0	H	0	0	0	
+	H	0	0	+	+	0	
+	0	0	0	0	0	+	
+	0	0	0	0	0	0	
+	+	0	+	0	0	+	Lesions in adventi- tia of aorta
0	0	0	0	0	0	+	
0	0	+	0	0	0	0	
H	0	0	0	+	+	0	Hemorrhages in small intestine
0	0	+	0	0	0	0	Hemorrhages in stomach
+	0	+	+	0	0	+	Lesions in aorta; miliary abscesses in lungs
+	0	0	0	0	+	+	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	+	0	0	0	0	
0	0	H	0	0	0	0	
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	Died in 10 minutes
0	0	0	0	0	0	0	
+	0	+	0	0	0	+	
+	0	0	0	0	0	0	
+	0	0	0	+	+	+	
0	0	0	0	0	0	0	
0	0	0	0	0	0	+	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	Fibrinous perito- nitis, organizing
+	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
+	0	+	0	0	0	0	
+	0	0	0	0	0	+	
0	0	+	0	0	0	0	
0	0	0	H	0	0	0	
0	0	0	0	0	0	+	Focal encephalitis ("chorea"). Les- ions in adventitia of aorta
+	0	0	+	0	0	+	
+	0	0	0	0	0	0	
+	H	+	H	0	0	0	
+	0	+	H	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	+	0	0	0	0	0	
+	0	0	0	0	0	+	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
+	0	0	H	0	0	+	
0	0	0	+	0	0	0	Lesions in media of aorta

TABLE 4—Continued

## STREPTOCOCCUS MITIS

Strain	Source	Days Cultivated	Rabbit	Dose in c.c.	Duration of Infection, Days	Died or Chloroformed*
39	Feces.....	3	124	30	7	0
			125	5	7	0
			126	10	7	0
			127	4	7	0
41	Feces.....	3	132	30	7	0
			133	10	7	0
			134	8	7	0
			135	5	7	0
43	Feces.....	3	140	30	7	0
			141	10	7	0
			142	8	7	0
			143	5	7	0
63	Blood culture (malignant endocarditis)	7	161	6	2	+
			162	5	3	0
			158	7	1	+
			167	5	2	+
93	Ankle joint (chronic deforming arthritis)	3	185	5	2	+
			186	5	2	+
			187	5	2	+
			188	5	7	0
			189	5	7	0

\* See Table 1 for the significance of + and H.

Since this is the only lesion occurring in rheumatic fever which appears to be absolutely specific, it is important to learn whether streptococci of the type cultivated from cases of rheumatism by Poynton and Paine will produce such lesions in rabbits, and especially whether the ability to produce these lesions is peculiar to this type of streptococci.

Bracht and Wächter<sup>56</sup> inoculated streptococci from two cases of rheumatism in which Aschoff-Geipel bodies were found, into a small series of rabbits. Some of the animals developed areas of focal myocarditis composed mainly of lymphocytes; and, altho the Aschoff type of lesion did not occur, they considered it significant that the lesions were of this more or less chronic type rather than in the form of the miliary abscesses which they obtained with *S. pyogenes*. Jackson<sup>57</sup> described myocardial lesions occurring in rabbits inoculated with streptococci from the Chicago milk-borne epidemic of sore throat, and with *S. viridans* from cases of endocarditis. She noted the presence of lesions composed of large mononuclear cells, and in the oldest lesions giant cells, and she considered the condition to be markedly similar to rheumatic myocarditis. Staining with pyronin-methyl green is not mentioned. It is to be noted that the sore-throat streptococci were hemolytic, and that altho they presented cer-

<sup>56</sup> Deutsch. Arch. f. klin. Med., 1909, 96, p. 493.

<sup>57</sup> Jour. Infect. Dis., 1912, 11, p. 243.

TABLE 4—Continued

## STREPTOCOCCUS MITIS

Heart - Muscle	Heart Valves	Joints	Voluntary Muscles	Lymph Nodes	Spleen	Kidneys	Remarks
0	0	0	0	0	0	0	
0	0	+	0	0	0	0	
+	0	+	0	0	0	0	
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	H	0	+	0	
0	0	0	0	0	0	0	
+	H	0	0	+	0	+	
0	0	0	H	+	0	0	
+	H	0	0	0	+	+	
0	+	0	0	0	0	+	
0	0	0	0	0	0	0	

tain peculiarities, they could not be differentiated by sugar tests from *S. pyogenes*. (The carbohydrate-fermentation reactions of milk-epidemic sore-throat streptococci were studied by Smith and Brown.<sup>58</sup>) Thalheimer and Rothschild<sup>59</sup> studied heart lesions in rabbits inoculated with *S. viridans*, with Poynton and Paine's strains of *S. rheumaticus*, and with strains of the epidemic sore-throat streptococci (the latter two had been cultivated some time). They described various types of myocarditis, among others areas of small-round-cell infiltration, as described by Bracht and Wächter, and also proliferative lesions, in which there was primarily a degeneration of muscle fibers, "but fibroblasts soon become prominent and develop rapidly to a fibrous stage." These latter they believed did not resemble Aschoff-Geipel bodies because "they show no predilection for a subendocardial situation nor for the bases of the cardiac valves," because giant cells were absent, and because the cells of which they were composed did not stain so deep a red with pyronin as do the Aschoff cells. Hartzell and Henrici<sup>60</sup> described myocardial changes of various kinds occurring in rabbits inoculated with strains of *S. viridans* from dental abscesses and pyorrhea alveolaris. In 2 of these rabbits there developed lesions of a proliferative character, in which multinucleated giant cells were prominent, and which they believed were similar to Aschoff-Geipel bodies.

<sup>58</sup> Jour. Med. Research, 1915, 31, p. 455.

<sup>59</sup> Jour. Exper. Med., 1914, 19, p. 429.

<sup>60</sup> Jour. Am. Med. Assn., 1915, 64, p. 1055.

TABLE 5  
STREPTOCOCCUS SALIVARIUS

Strain	Source	Days Cultivated	Rabbit	Dose in c.c.	Duration of Infection, Days	Died or Chloroformed*
5	Cervical lymph node.....	3	26	3	5	0
			27	5	5	+
			28	15	3	0
			29	45	1	+
9	Tonsil.....	7	41	5	1	0
			42	5	2	0
			43	5	3	0
			44	5	3	0
			45	5	3	0
			46	5	3	0
19	Pyorrhea alveolaris.....	4	53	5	4	0
			54	5	4	0
			55	5	4	0
			56	5	4	0
25	Dental abscess.....	8	78	5	6	0
			79	5	6	0
			80	5	6	0
			81	5	6	0
35	Strain 19 after one animal passage	3	115	45	2	+
			116	5	2	0
			117	5	1	+
			118	5	2	+
56	Feces.....	11	171	5	8	0
			172	8	8	0
			173	5	8	0
			174	10	9	0
64	Blood culture (malignant endocarditis)	7	175	5	8	0
			177	10	8	0
			178	5	8	0
			179	8	8	0
95	Dental abscess.....	1	202	3	3	0
			203	5	1	+
			204	8	1	+
24	Tonsil.....	8	73	75	2	0
			74	30	6	0
			75	5	6	0
			76	5	6	0
			77	5	2	0

\* See Table 1 for the significance of + and H.

The heart lesions which occurred frequently in the rabbits of this study varied widely in character, presenting but one feature in common, namely, their focal nature. There were small areas of necrosis in the muscle fibers, frequently containing masses of cocci, and at times unaccompanied by other changes save for interstitial hemorrhages; then there occurred, in addition to this condition, varying degrees of polymorphonuclear infiltration, up to the development of well-marked miliary abscesses. These lesions we may classify in one group as degenerative-exudative.

TABLE 5—*Continued*  
STREPTOCOCCUS SALIVARIUS

Heart Muscle	Heart Valves	Joints	Voluntary Muscles	Lymph Nodes	Spleen	Kidneys	Remarks
0	0	+	0	0	0	0	Osteomyelitis of tibia Bronchopneumonia
+	0	0	0	+	+	0	
0	0	0	0	+	0	+	
0	0	0	H	+	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	+	0	0	0	+	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
+	0	0	0	0	0	0	
0	0	+	0	0	0	0	
0	0	0	H	0	0	0	
+	+	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	+	0	0	0	0	Subacute meningitis ("chorea")
+	+	+	0	+	0	+	
+	+	+	0	0	0	+	
0	0	0	0	+	+	0	
0	+	+	0	0	0	+	
0	0	+	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	Subacute meningitis ("chorea")
0	0	0	0	0	0	+	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
0	H	+	H	0	0	0	Subacute meningitis ("chorea")
0	0	0	0	0	0	0	
0	0	H	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	

A second group consists of lesions in which lymphocytes, with occasionally plasma cells, were the principal or only infiltrating cells. Here there was little evidence of degeneration. The cells collected at times about the vessels, more frequently in spaces between the muscle fibers. They occurred in all parts of the heart.

The third group is composed of lesions that were essentially proliferative in character. Here, as in the case of the first type, the lesions varied widely. What we consider to be the acute stage of this condition consisted of an area

TABLE 6  
STREPTOCOCCUS FÆCALIS

Strain	Source	Days Cultivated	Rabbit	Dose in c.c.	Duration of Infection, Days
3	Saliva.....	3	18	1	2
			19	5	2
			20	15	2
			21	45	2
40	Feces.....	3	128	30	7
			129	10	7
			130	8	7
			131	5	7
42	Feces.....	3	136	30	7
			137	10	7
			138	8	7
			139	5	7
62	Dental abscess.....	6	162	9	3
			163	6	3
			164	5	3
			165	5	3
70	Blood culture (malignant endocarditis).....	3	180	10	1
			181	10	1
			182	10	1
			183	10	1
			184	10	1

\* See Table 1 for the significance of + and H.

of necrosis in the muscle fibers. Surrounding the dead muscle cells was an aggregation of large round or more commonly oval and spindle-shaped cells, containing large clear nuclei and having an abundant protoplasm, which tended to take the basic stains. These cells were very probably of fibroblastic origin; in fact, in some of the lesions there were a number of cells which looked like fibroblasts that showed mitotic figures. These cells stained red with pyronin-methyl green (the tissues were all fixed in Helley's fluid). Where necrosis was present there were also usually a few polymorphonuclear leukocytes, and in a few instances we found small abscesses in the heart muscle surrounded by a wide zone of cells such as have just been described. More commonly, however, there was no necrosis, and the large cells were collected between the muscle fibers, spreading them apart and thus forming fusiform masses. They also in places collected about vessels, the cells apparently being derived from the adventitia of the latter, and here the lesions could not be differentiated from those typical of rheumatic fever as described by Aschoff. These proliferative lesions were noted frequently beneath the endocardium and pericardium, and especially near the bases of the valves. In the latter situation multinucleated giant cells were particularly frequent, altho they were noted in other areas also. Many of the subendocardial lesions contained masses of hyaline material, probably altered fibrin.

In short, we repeatedly produced in rabbits, by the inoculation of streptococci, lesions which could not be histologically differentiated from the type of myocarditis generally accepted as specific for rheu-

TABLE 6—*Continued*

STREPTOCOCCUS FÆCALIS

Died or Chloroformed*	Heart Muscle	Heart Valves	Joints	Voluntary Muscles	Lymph Nodes	Spleen	Kidneys
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	H	H	0	0	0
0	0	0	0	H	0	0	0
0	0	0	+	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	+	0	0	0	0
0	+	0	+	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	+	0	0	0	0
0	0	0	0	0	0	0	0
0	+	0	+	+	0	0	+
0	+	0	+	0	0	0	0
0	+	+	0	0	0	0	+
0	+	0	0	0	0	0	+
+	0	0	0	0	0	0	0
+	0	0	0	0	0	0	0
+	0	0	0	0	0	0	0
+	+	0	0	0	0	0	0
+	0	0	0	H	0	0	0
+	0	0	0	H	+	0	0



Fig. 4. Subendocardial focus of proliferative myocarditis, showing fibrin.

matic fever. Some of the lesions were atypical in the occurrence of considerable necrosis and the admixture of other types of inflammatory cells; perhaps these cells did not stain so deep a red with pyronin as do those in the human heart. We hold that these differences are not so great but that they can be explained by differences in the degree of infection between the inoculated rabbit and the man infected with the rheumatic virus, or difference in the constitution of the cells as they occur in rabbits and in man. In the general appearance of the cells, their basicity, their accumulation in fusiform masses between the fibers or in rosettes about the vessels, their frequent subendocardial position, and the frequent occurrence of multinucleated giant cells, these lesions presented all the characteristics of the typical Aschoff-Geipel nodules.

In Table 7 are indicated the percentages of rabbits which developed these different types of myocarditis after inoculation with the various strains of streptococci.

TABLE 7

THE PERCENTAGES OF RABBITS WHICH DEVELOPED DIFFERENT TYPES OF MYOCARDITIS AFTER INOCULATION WITH VARIOUS STRAINS OF STREPTOCOCCI

	Exudative %	Lympho- cytic %	Prolifer- ative %	Total %
<i>S. pyogenes</i> , Group I.....	5	8	2	15
<i>S. pyogenes</i> , Group II.....	15	10	15	40
<i>S. anginosus</i> .....	8	7	4	19
<i>S. mitis</i> .....	6	10	15	31
<i>S. salivarius</i> .....	5	8	0	13
<i>S. faecalis</i> .....	5	9	14	28
Hemolytic strains.....	9	9	7	25
Nonhemolyte strains.....	5	9	10	24

It will be seen from Table 7, that while variations occur, they are not sufficiently marked to establish specificity. Especially when the hemolytic strains are compared with the viridans group, do the differences become slight; and it is noteworthy that the totals indicate that neither group has a greater affinity for the heart muscle than the other.

*Aorta*.—Klotz<sup>61</sup> described fatty changes and connective-tissue increase in the intima of the arch of the aorta of rabbits repeatedly inoculated with streptococci of low virulence. In later papers<sup>62</sup> he described lesions of the arch of the aorta which are constantly present in rheumatic fever. These consisted of small foci of lymphocytic and

<sup>61</sup> Brit. Med. Jour., 1906, 2, p. 1767.

<sup>62</sup> Tr. Assn. Am. Phys., 1912, 27, p. 181. Jour. Path. and Bacteriol., 1913, 18, p. 259.

plasma-cell infiltration about the vessels of the adventitia and in the outer part of the media; in one case an acute aneurysm developed. These findings led me to examine sections of the arch of the aorta in my rabbits.

Inflammatory changes were found in the aorta in 4 animals, all of which had been inoculated with strains of *S. mitis*. In 2 there was a general infiltration of the adventitia with large cells such as those observed in the heart muscle; within this area of proliferative change miliary abscesses occurred. The media was not involved, nor did the reaction bear any relation to the vasa vasorum. The lesions began at the aortic valve and did not extend beyond the ascending limb. In one of the rabbits there was also a small area of similar infiltration beneath the intima, just above the valve cusp. A third rabbit had a small collection of lymphoid cells in the adventitia.

Hartzell and Henrici<sup>60</sup> described lesions in the inner part of the media of 3 rabbits inoculated with streptococci from dental abscesses. These lesions consisted of an area of necrosis, with broken and twisted elastic fibrils, containing a few lymphocytes. The fact that the lesions were found in young animals and were not found in a series of controls led them to suggest that possibly they were due to the inoculated streptococci.

The vascular lesion in the fourth rabbit I consider to be an earlier stage of this process. There was here a similar destruction of tissue in the media, just beneath the intima, but the area contained a number of large mononuclear cells with some multinucleated giant cells. These cells also stained red with pyronin. A section was submitted to Dr. Klotz, who suggested that these cells were phagocytes called out by the necrotic elastic tissue fibrils. The close resemblance of this lesion to the Aschoff-Geipel nodules which were produced in the heart muscle by this same strain leads me, however, to believe that here I had a localization of the streptococci in the media.

*Joints.*—Arthritis has been produced in rabbits by Poynton and Paine,<sup>21</sup> Beattie,<sup>31</sup> Shaw,<sup>51</sup> Meyer,<sup>63</sup> Wassermann,<sup>34</sup> and Rosenow<sup>64</sup> with the streptococcus from cases of rheumatic fever, and has been considered by these observers to be evidence of the specific etiologic relation of this organism to that disease.

Cole,<sup>36</sup> however, pointed out that arthritis might frequently be produced by other streptococci; but Beattie<sup>65</sup> considered that the experimental arthritis produced by *S. rheumaticus* is different from that caused by *S. pyogenes* in that it is usually nonpurulent; he also found that this experimental arthritis resembled human rheumatism in that the condition tended to pass quickly from joint to joint and was aggravated by exposure to cold.<sup>31</sup> Poynton and Paine cultivated their "diplococcus" from cases of chronic deforming arthritis, and were able to produce a chronic form of osteo-arthritis in rabbits by inoculation.

Davis<sup>66</sup> observed that arthritis developed somewhat regularly in rabbits injected with hemolytic streptococci, but was seldom produced by strains of

<sup>63</sup> Deutsch. med. Wchnschr., 1901, 27, p. 81.

<sup>64</sup> Jour. Infect. Dis., 1914, 14, p. 61.

<sup>65</sup> Jour. Exper. Med., 1907, 9, p. 186.

<sup>66</sup> Jour. Am. Med. Assn., 1912, 58, p. 1852.



Fig. 5. Lesion in adventitia of aorta.



Fig. 6. Focus of proliferative inflammation in media of aorta.

*S. viridans*. Thalheimer and Rothschild,<sup>97</sup> however, found that arthritis was caused in half of their rabbits by *S. viridans*.

Coombs<sup>98</sup> noted the occurrence of "submiliary nodules," similar to those occurring in the heart muscle, in the articular tissues of cases of rheumatic fever; and Coombs, Miller, and Kettle<sup>99</sup> produced similar lesions in rabbits by inoculation with *S. rheumaticus*. Jackson<sup>100</sup> made studies of the histologic changes in the joints of rabbits produced by various streptococci, and observed similar proliferative changes. She states that "the differences in the inflammatory process in the joints of 17 rabbits of this series studied at periods varying from two hours to four months are only such as are consistent with the varying phases of a single inflammatory process, nor are there any striking differences in the reaction produced by the various kinds of streptococci employed."

Arthritis was the lesion most frequently observed in our series. In some cases there was pronounced congestion of the capsule, with hemorrhages where exudate was not present in the joint cavity; hemorrhages were especially frequent beneath the periosteum of the head of the tibia. Where arthritis was diagnosed, there was an exudate within the joint. This usually consisted of a thick mucous fluid which was turbid. Microscopical examination showed that the cloudiness was due to polymorphonuclear leukocytes. Then there were observed all degrees of severity in this condition. As the exudate increased in amount, distending the joints, it became cloudier and took on the appearance of ordinary pus. Where still more extensive involvement occurred, there was apparently considerable fibrin in the exudate, so that the joint cavities became filled with semisolid cheesy-looking material. Arthritis developed most frequently in the knee joints, next most frequently in the elbows, and occasionally in the shoulders and small joints of the paws (the latter were not examined routinely; for that reason our figures may be somewhat low). Frequently more than one joint was involved. Extensive histologic studies were not made, but sections were taken from some of the more pronounced lesions. These showed an exudation of polymorphonuclear cells in the synovial tissues, with a similar exudate on the joint surfaces. In one instance there were found multiple minute abscesses in the marrow of the tibia.

There were no pronounced differences between the percentages of arthritis produced by the various classes of streptococci, nor were there observed any differences in the character of the exudate. Joint lesions were produced with equal frequency by both hemolytic and nonhemolytic strains.

*Voluntary Muscles.*—Rosenow<sup>64</sup> called attention to lesions in the voluntary muscles produced by the inoculation of streptococci, and described similar lesions in man in cases of "muscular rheumatism" from which streptococci were isolated.

The muscle lesions in my rabbits were most frequent in the extremities, but in some instances they involved all the muscles, including the diaphragm.

<sup>97</sup> Jour. Exper. Med., 1914, 19, p. 444.

<sup>98</sup> Lancet, 1912, 2, p. 1209.

<sup>99</sup> Jour. Infect. Dis., 1913, 12, p. 364.

There were 2 types of lesions. The first group was composed of interstitial hemorrhages, and these were the more frequent. They usually occurred in circumscribed areas, but in two instances were very extensive, involving all the muscles of one extremity. The second group of lesions was rather peculiar. Grossly these appeared as small short white streaks running parallel with the muscle fibers, best seen when the rabbit was freshly killed and while the muscles still retained their translucent characters. Microscopically, these areas showed scattered isolated necrotic muscle fibers surrounded by a narrow zone of large somewhat flattened cells. There was usually no leukocytic infiltration, and this would seem to indicate that the condition was due to circulating toxins rather than to a localization of the streptococci. In a more pronounced stage of the condition a number of neighboring fibers were involved, and here the cellular reaction was more marked. The fibers were surrounded by

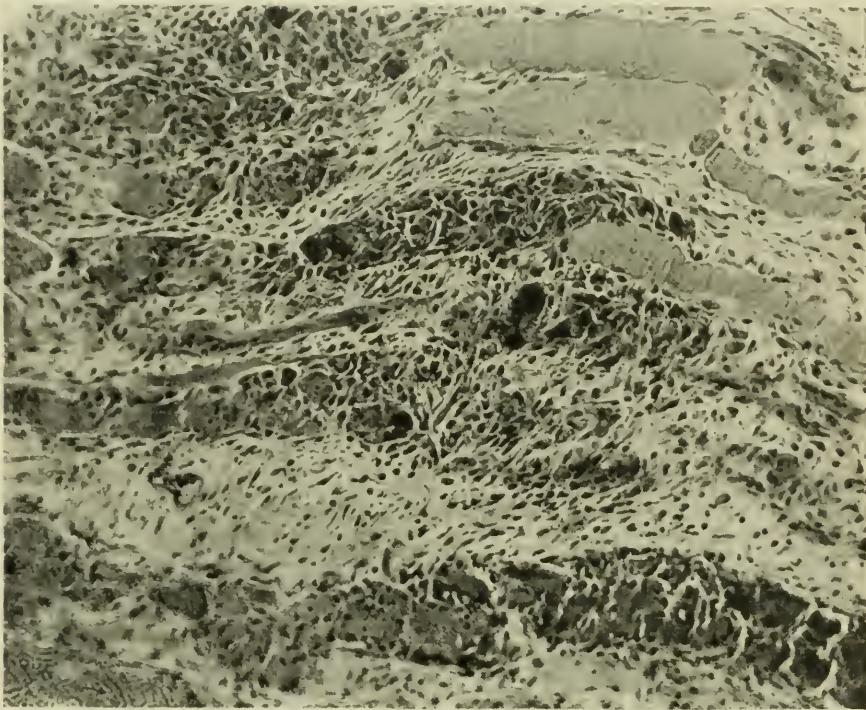


Fig. 7. Lesion in voluntary muscle, showing the breaking up of necrotic fibers and proliferative inflammation.

numbers of large oval or spindle-shaped cells. The histologic interpretation is complicated by the peculiar changes which followed the degeneration of muscle fibers, which have been described by Dawson.<sup>70</sup> Thus it becomes difficult to say which of the cells were derived from the muscles and which were of fibroblastic origin or were wandering endothelial cells. Many of the cells were in mitosis. The presence of muscle giant cells was an added striking feature. Some of the necrotic muscle fibers appeared granular and stained deeply with hematoxylin, suggesting an early deposition of lime salts. In still more marked lesions the changes described were accompanied by a leukocytic infiltration leading to the development of multiple minute abscesses.

<sup>70</sup> Jour. Path. and Bacteriol., 1909, 13, p. 174.

*Lymphadenoid Tissues.*—A number of the rabbits at autopsy showed one or more of the lymph nodes to be swollen and deeply congested or hemorrhagic. The popliteal nodes were most frequently involved, and next in frequency were the axillary glands. This is probably due to the frequency of arthritis in the knees and elbows, altho such swollen glands have been observed in the absence of any grossly visible arthritis. In some instances practically all the nodes, including the mesenteric, were involved.

Microscopically there was noted intense congestion, usually with marked hemorrhage, and pronounced changes in the parenchyma—degeneration of lymphocytes with karyolysis, a migration of cells from the adenoid tissue into the sinuses, and a disappearance of the follicles. In addition there was a pronounced increase of the endothelial cells, large numbers of which lay free in the sinuses and were ingesting nuclear fragments.

The spleen showed somewhat similar changes, save that intense congestion, hemolysis, and the deposition of fibrin were striking features; but there were noted the same tendency for the follicles to disappear, and the same increase of phagocytic endothelial cells. The condition was identical with the acute splenitis which accompanies septic processes in man. In several instances there was noted a deposition of hyaline material in the germinal centers. There were also observed several spleens containing colonies of cocci in many areas. One rabbit with endocarditis showed septic infarcts in the spleen.

In 2 rabbits inoculated with the same strain there was marked congestion with multiple punctate hemorrhages in the thymus. These rabbits had also acute splenitis and lymphadenitis. No parenchymatous changes were noted in the thymus, however.

*Kidneys.*—Various types of kidney lesions have been described by several authors studying experimental streptococcal infections in rabbits. Le Count and Jackson<sup>71</sup> made a very thorough study of these changes, and as the lesions which I found were identical in every respect with theirs, a very brief description will suffice.

While there occurred at times minute polymorphonuclear abscesses with or without the presence of bacterial emboli, the great majority of the lesions consisted of areas of lymphocytic infiltration. These were most frequently present in the cortex, and almost invariably occupied a perivascular position, either as a small nodule surrounding one of the smaller vessels, or as long radiating streaks running through the cortex at right angles to its surface. In a number of instances there was noted infiltration about the glomeruli, at times of lymphocytes, but also frequently of larger mononuclear cells tending to assume the spindle shape of the cells found in the heart and voluntary-muscle lesions. Such cells were arranged concentrically about the glomerulus.

I realize that kidney lesions of this character may occur spontaneously in rabbits, and I attempted to throw these out of my figures by not accepting any lesions where fibrosis or dilatation of tubules had occurred, since it is doubtful if such changes could develop in the streptococcal lesions in the short time these rabbits were allowed to live.

<sup>71</sup> Jour. Infect. Dis., 1914, 15, p. 389.



Fig. 8. Kidney lesion, showing a streak of lymphocytic infiltration extending across the cortex.

Kidney lesions were produced somewhat more frequently by the hemolytic strains than by *S. viridans*, but there was not observed any greater tendency of the former group to produce miliary abscesses.

*Other Tissues.*—A great variety of inflammatory changes was observed in the liver, but my rabbits were so constantly infected with coccidia that I did not feel safe in attributing any of these lesions to the streptococci. No changes were seen in the gallbladder save the results of coccidiosis. In several instances I noted punctate hemorrhages in the wall of the stomach, in one case associated with a small ulceration; microscopically, this showed free blood in the submucosa and a loss of substance in the overlying mucous membrane, with no inflammatory reaction. Two animals developed punctate hemorrhages in the submucosa of the appendix, and in one there were similar lesions in the small intestine. I repeatedly noted in sections of the appendix areas of necrosis in the follicles of the submucosa, such as have been described by Poynton and Paine<sup>71</sup> and by Rosenow.<sup>72</sup> but these always were associated with the presence of coccidia. One rabbit developed bronchopneumonia, and another showed miliary abscesses throughout the lungs.

#### ANALYSIS AND DISCUSSION OF RESULTS

Charts 1 to 3 also present the results of the experiments. These charts require a word of explanation. The column labeled "Infected" indicates the total percentage of animals which showed some evidence

<sup>72</sup> Jour. Infect. Dis., 1915, 16, p. 240.

that the inoculation resulted in infection, either in death or in the occurrence of localized lesions or both. The column marked "Died" shows the number of rabbits that died spontaneously within the time limits of the experiments. The "Septicemia" column denotes the percentage of rabbits that died spontaneously without developing lesions, usually within 24 hours; here septicemia was undoubtedly the cause of death. It is not to be inferred that these were the only rabbits that developed septicemia, as probably many of the animals which had localized lesions also had a blood infection; certainly this is true of the animals that developed splenitis.

It will be seen from Chart 1 that practically all of the various lesions were produced by one or another strain of each of the types of streptococci in the classification used. The only variations, then, were quantitative ones, and such variations were considerable. However, in order to establish the value of such quantitative differences between the various strains of streptococci in point of tissue affinity and virulence, conclusions should be drawn from a considerably larger series of experiments than is here reported. That is, while the whole number of strains and of rabbits studied was fairly large, these numbers become small in the individual groups, and, moreover, there was a disparity between the numbers in some groups and those in others. Thus Group I of *S. pyogenes* was represented by 11 strains that were inoculated into 46 rabbits; Group II, by 5 strains and 20 rabbits; *S. anginosus*, by 6 strains and 26 rabbits; *S. mitis* by 17 strains and 74 rabbits; *S. salivarius*, by 9 strains and 38 rabbits; and *S. faecalis*, by 5 strains and 21 rabbits.

The quantitative variations, probably for the reasons just given, having occurred in a sort of "hit or miss" manner, do not lend themselves readily to analysis, especially since each of the groups has been classified by the use of 2 characteristics, their hemolytic powers and their ability to ferment carbohydrates. We may study the influence of each of these factors separately by combining those strains fermenting the same carbohydrates, irrespective of hemolysis, and vice versa.

In Chart 2 is shown the relation of the power of sugar-fermentation to virulence and elective organ affinities. The 1st group, consisting of strains fermenting both salicin and mannite, contains *S. pyogenes*, Group II, and *S. faecalis* of the former classification; this group represents 10 strains and 41 rabbits. The 2nd group is composed of strains fermenting salicin alone, and contains *S. pyogenes*, Group I, and *S. mitis*. This group contains the majority of strepto-

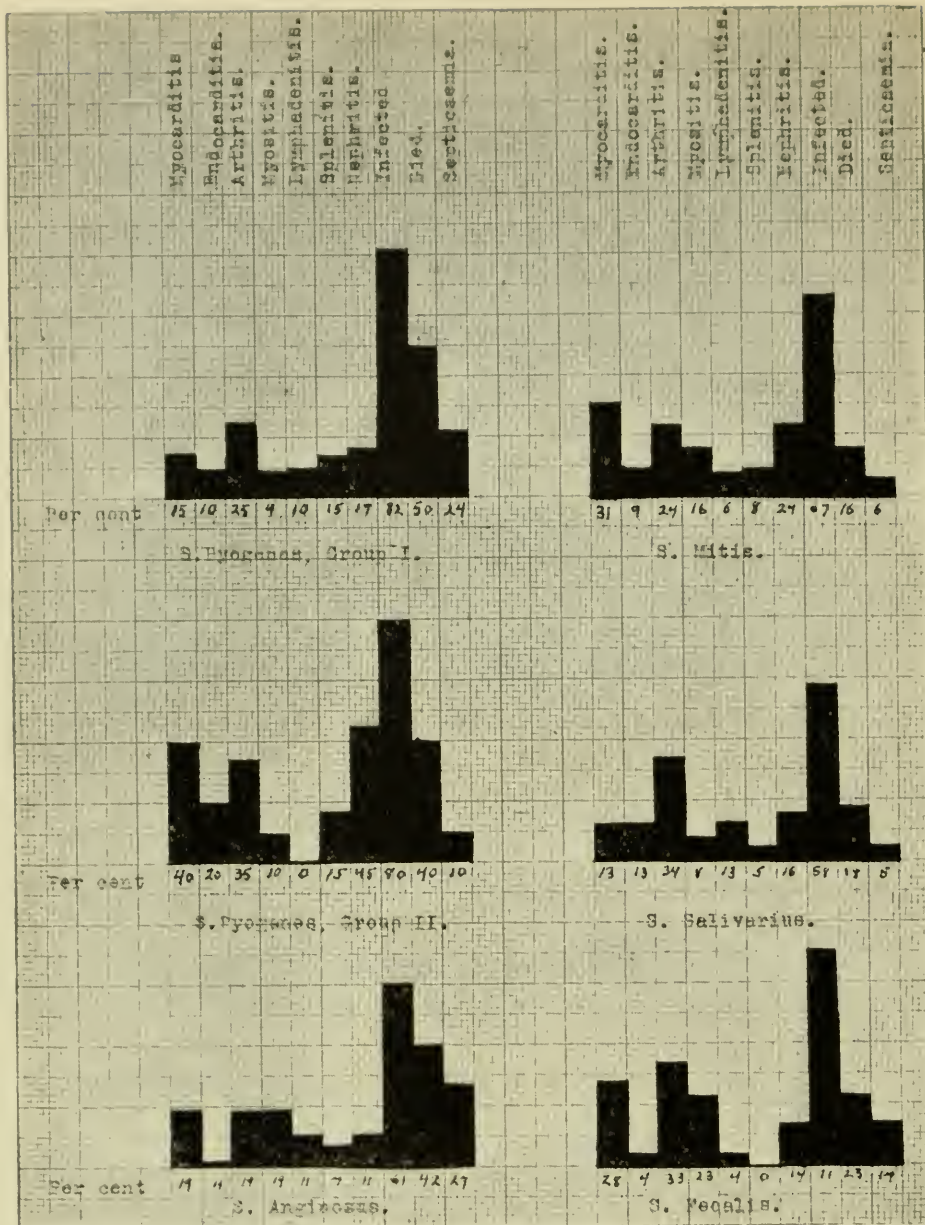


Chart 1. A comparison of the total percentages of rabbits inoculated as indicated, that showed (1) lesions in various organs, (2) evidence that the inoculation resulted in infection (either by death or the occurrence of localized lesions or both), (3) that died spontaneously within the limits of the experiment, and (4) that died of septicemia, without developing lesions.

cocci pathogenic for man. There were 28 strains of the second group inoculated into 120 rabbits. The last group, fermenting neither of the two carbohydrates, contains *S. anginosus* and *S. salivarius*; this group is composed of 15 strains which were injected into 64 rabbits.

In this chart the number of observations in each group is larger and it will be seen that pronounced variations do not occur. Espe-

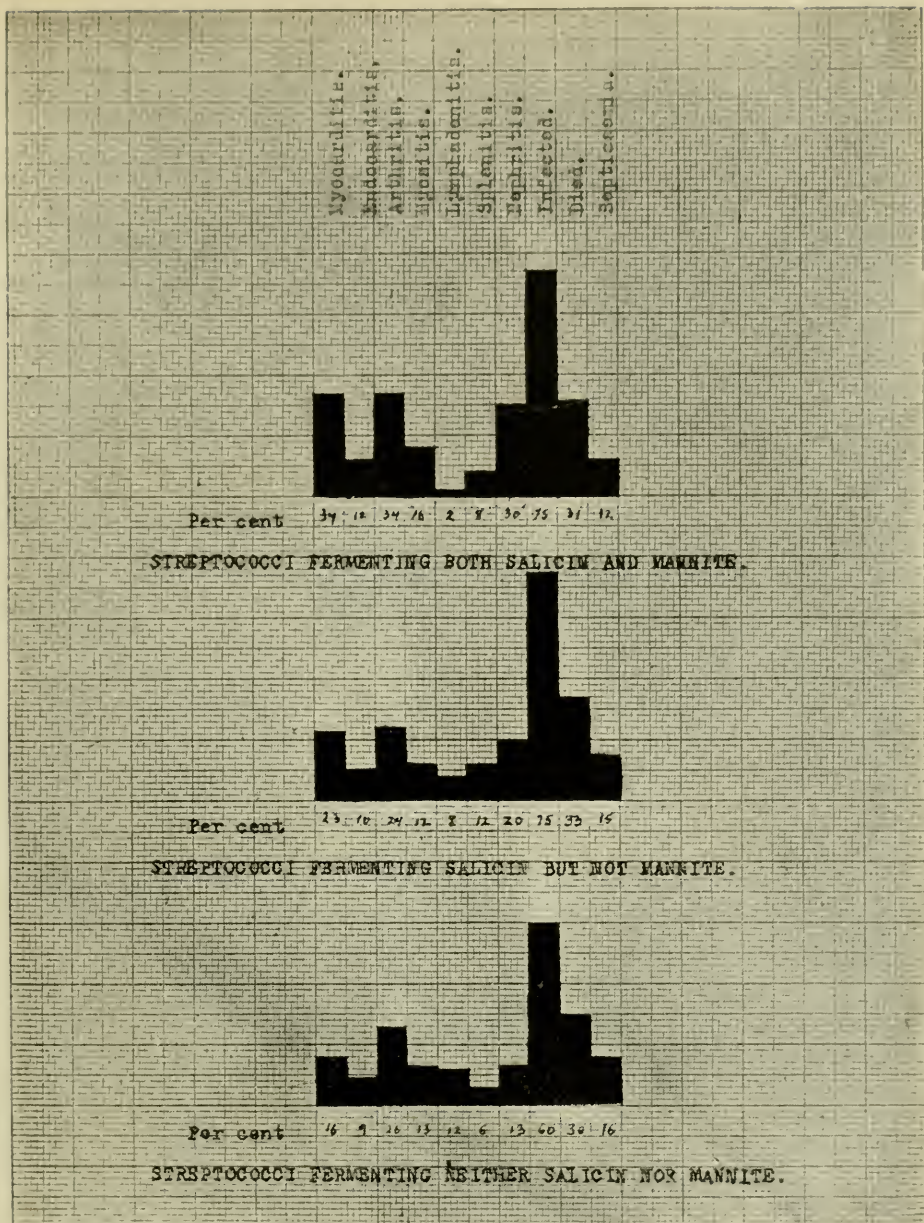


Chart 2. The relation of the sugar-fermenting power to virulence and elective organ affinities.

cially the virulence, as measured by the percentages of spontaneous deaths and septicemias, remains practically constant. There is a slight tendency to a decrease in all the localized infections (especially in the myocardial and renal lesions) as we pass from the 1st to the 3rd groups. This variation, however, is not pronounced and its significance is doubtful.

Chart 3 presents the results obtained when the hemolytic strains are compared with the nonhemolytic irrespective of the sugar tests.

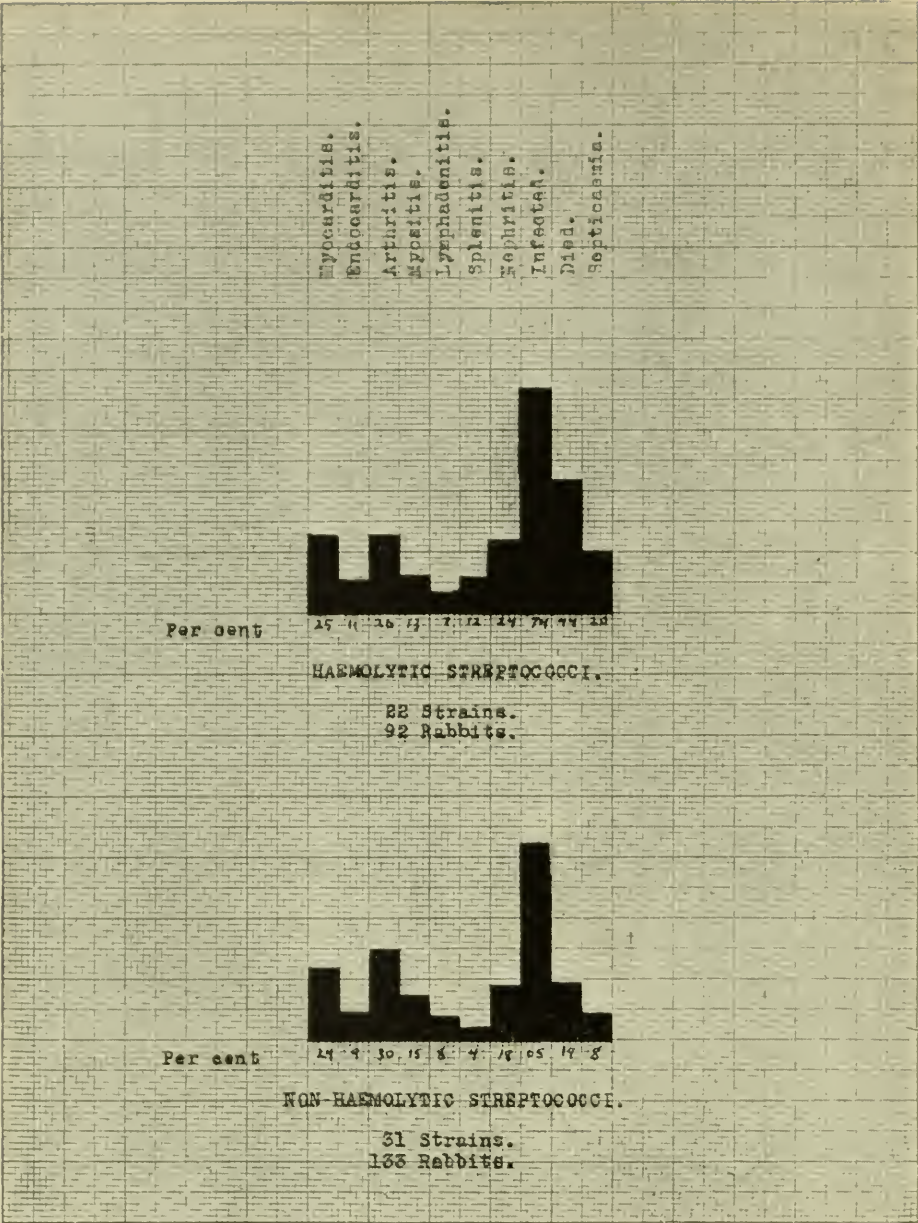


Chart 3. The relation of the hemolytic power of streptococci or the lack of it to virulence and elective organ affinities.

This chart shows two striking features. The hemolytic strains are far more virulent, as shown by the much higher percentages of spontaneous deaths and of septicemias, and also by the much greater incidence of acute splenitis. The other figures, denoting the occurrence of localized lesions, remain practically the same in the two groups.

The figures in the first four columns, denoting the incidence of the so-called rheumatic lesions, are particularly significant, for they indi-

cate that these lesions are produced with practically identical frequency by both *S. hemolyticus* and *S. viridans*. This is not in accord with what we have been led to believe from previous literature, nor with clinical experience. For certainly we find nonhemolytic strains associated with endocarditis, at least, far more frequently than we do hemolytic ones. It is suggested that the lesions produced in rabbits may be no indication of the tissue affinities of the same strains in man.

#### SUMMARY AND CONCLUSIONS

Fifty-three strains of streptococci from various sources were inoculated into 225 rabbits, and the virulence and elective organ affinities were compared with the powers of hemolysis and carbohydrate-fermentation.

The carbohydrate-fermentation tests are of no significance from the standpoint of virulence, and of slight or doubtful significance from the standpoint of tissue localization.

The hemolytic streptococci are more virulent than the non-hemolytic, but the two classes localize in the same tissues with equal frequency.

We are not justified, from evidence obtained by rabbit-inoculation experiments, in recognizing any particular class of streptococci as specific for rheumatic fever, since the various rheumatic lesions, arthritis, myocarditis, endocarditis, and myositis, may be produced by some strains in each of the varieties, and are produced in equal proportion by both hemolytic and nonhemolytic streptococci.

Streptococci of various kinds may produce in rabbits types of myocarditis which cannot be differentiated from the Aschoff-Geipel nodules generally considered diagnostic of rheumatism.

Experimental streptococcal endocarditis usually develops by implantation rather than by embolism.

# THE CORRELATION OF CERTAIN REACTIONS OF COLON BACILLI AND LIKE ORGANISMS WITH SOURCE \*

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The colon-aerogenes group of bacteria has claimed the interest of bacteriologists from the discovery of the first member of the group until the present time, altho opinion as to the exact meaning of their presence in water and milk has changed somewhat. Since these organisms are known to occur in the intestines of all animals, their presence in either milk or water was at first regarded as an evidence of fecal contamination. Later Prescott and Winslow<sup>1</sup> regarded the number of the organisms present as more significant than their mere presence. Be this as it may, since there is always a possibility that they may indicate pollution, it is of advantage to have quick methods of determining whether or not organisms present in milk or water belong to the colon group, and if possible their source.

Much work has been done on the classification of the group in England, Germany, and America altho most of this work has been along the line of differentiating different members of the group by their powers of fermentation in carbohydrate media. MacConkey<sup>2</sup> finally narrowed his classification down to 4 groups, separated from each other on the basis of their power to ferment saccharose and dulcitol. In connection with their fermenting powers he considered other characteristics, such as gelatin-liquefaction, indol-production, motility, and the Voges-Proskauer<sup>3</sup> reaction. Twort,<sup>4</sup> Vorlond,<sup>5</sup> and Winslow and Walker<sup>6</sup> also differentiated this group on the basis of their fermenting powers in carbohydrate media.

Bergey and Deehan<sup>7</sup> isolated a large number of organisms from milk, kefir, and sewage, and studied them with a view to classifying them according to the scheme of MacConkey. They divided the whole group into 16 subgroups according to ability to ferment saccharose, dulcitol, adonitol, and inulin, and each of these again into 16 smaller groups according to motility, gelatin-liquefaction, indol-production, and the Voges-Proskauer reaction. It

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<sup>1</sup> Elements of Water Bacteriology, 1915, p. 149.

<sup>2</sup> Jour. Hyg., 1905, 5, p. 333.

<sup>3</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1898, 28, p. 20.

<sup>4</sup> Centralbl. f. Bacteriol., R., 1907, 40, p. 508.

<sup>5</sup> Ibid., I, O., 1908, 45, p. 97.

<sup>6</sup> Science, 1907, 26, p. 797.

<sup>7</sup> Jour. Med. Research, 1908, 14, p. 175.

remained for Rogers, Clark, and Davis<sup>8</sup> to utilize a method first used by Keyes<sup>9</sup> to determine accurately the amount of gas evolved by different strains of colon bacilli and to arrange them into classes according to this.

Keyes found that colon bacilli grown on synthetic media and in vacuo produce a definite volume of gas in a given time and that the ratio of  $\text{CO}_2:\text{H}_2$  is constant. Rogers, Clark, and Davis repeated this work of Keyes and with slight modifications divided the colon bacilli into classes according to their  $\text{CO}_2:\text{H}_2$  ratios. They found that all the organisms fell into 3 groups: a high-ratio group, in which the  $\text{CO}_2:\text{H}_2$  ratio is 1.90 or above this; a low-ratio group, in which the ratio is about 1.06; and an  $\infty$  ratio group, in which only  $\text{CO}_2$  is evolved. Their next step was to make a series of investigations of like organisms from different known sources to see if those obtained from any one source could be definitely related to any one of these gas ratios. In their system of classification, the gas ratio formed the only basis of differentiation; members classed within a single group display marked differences in powers of liquefaction, indol-production, and motility, but all agree in gas ratios.

Rogers, Clark, and Evans<sup>10</sup> made a study of 150 strains from bovine feces and found that 149 of them showed a low gas ratio. Rogers studied systematically organisms like the colon bacilli, from surface water, and found that one-third of these organisms corresponded to the type found in human and bovine feces. Rogers, Clark, and Evans<sup>11</sup> in an extensive study of the colon group occurring on grains found that the majority of these organisms belonged to the high-ratio group and that none of those found on grain corresponds to those found in feces.

Routine gas analysis in vacuo, however, is too complicated and requires too much apparatus, presenting technical difficulties not easily overcome in the average bacteriologic laboratory, and so any detectable characteristic which could be found invariably accompanying gas-production would prove a boon. This was found by Clark and Lubs<sup>12</sup> in the use of two easily accessible indicators. They found that when the organisms, in producing gas, had reached the maximal amount, the medium had a certain  $\text{H}^+$  ion concentration and that this could be measured by the use of either methyl red or paranitrophenol (preferably methyl red), as this  $\text{H}^+$  ion concentration happens to be within the limits of their color changes. Paranitrophenol is colorless in a  $\text{H}^+$  ion concentration of about  $1 \times 10^{-5}$ ; in a concentration below this it gradually becomes colored till at a concentration of  $\text{H}^+ 1 \times 10^{-6}$  it is yellow-green. Methyl red is even more brilliant as an indicator, being bright red at a  $\text{H}^+$  ion concentration of  $1 \times 10^{-5}$  and changing to clear yellow at a  $\text{H}^+$  ion concentration of  $\text{H}^+ 1 \times 10^{-6}$ . The high-ratio cultures give a green color with paranitrophenol and a yellow color with methyl red, indicating that they are alkaline, while the low-ratio cultures give no color with paranitrophenol and a red color with methyl red, indicating that they are acid.

It was found by these investigators that only those organisms isolated from feces produce this low ratio, so that the sanitary significance is easily seen, it being only necessary to plant the organisms in the peptone dextrose dihydrogen-phosphate medium, incubate for 5 days at 30 C., and then determine the

<sup>8</sup> Jour. Infect. Dis., 1914, 14, p. 411.

<sup>9</sup> Jour. Med. Research, 1909, 21, p. 69.

<sup>10</sup> Jour. Infect. Dis., 1914, 15, p. 99.

<sup>11</sup> Ibid., 1915, 17, p. 137.

<sup>12</sup> Ibid., p. 160.

H<sup>+</sup> ion concentration. Levine<sup>13</sup> in a recent paper correlated the Voges-Proskauer reaction with the H<sup>+</sup> ion concentration, having found that only those organisms that show a high gas ratio give the Voges-Proskauer reaction, and that these are rarely found in feces.

It might be interesting to note in this connection that at the meeting of the Society of American Bacteriologists in Urbana, Ill., in 1915, Winslow and Kligler<sup>14</sup> reported on the standard tests for colon bacilli, the committee, previously appointed, having decided on (1) titratable acidity, (2) H<sup>+</sup> ion concentration, (3) milk reactions, (4) indol-production, (5) gelatin liquefaction, (6) chromogenesis. This gives 3 large groups; (1) *B. coli*—coagulating milk, high H<sup>+</sup> ion concentration, indol-positive; (2) *B. aerogenes*—coagulating milk, low H<sup>+</sup> ion concentration, indol-negative; and (3) the *B. typhi* group—alkaline reaction, high H<sup>+</sup> ion concentration. There are 3 subgroups to Group 1 according to indol-production and sucrose-fermentation and 3 subgroups to Group 3.

A number of organisms which had been isolated and previously studied in this laboratory were selected for reinvestigation and these were studied according to the different methods.

#### TECHNIC

The fermentative powers were tried with lactose, sorbite, raffinose, dextrin, dextrose, saccharose, dulcitol, mannitol, maltose, adonitol, and inulin, a Hiss serum-water medium containing 1% of the various carbohydrates being used.

The Voges-Proskauer reaction was studied according to Levine's method, the Clark and Lubs medium\* for determining H<sup>+</sup> ion concentration being used, with an incubation of 48 hours, after which 5 c.c. of a 10% KOH solution were added. All cultures were kept 1 week as some of them became positive in that time.

For liquefaction of gelatin the gelatin was inoculated with a few drops of a broth culture and the level of the surface of the gelatin marked by a narrow strip of paper. The tube was allowed to stand 30 days. The liquefaction was measured in millimeters.

Indol-production tests were made by inoculating peptone and incubating for 7 days, then adding H<sub>2</sub>SO<sub>4</sub> drop by drop, and finally 1 c.c. of 0.01% NaNO<sub>2</sub> solution.

The indicator tests were made as described by Clark and Lubs.

The fermentative powers of colon bacilli from milk, which had been tried previously by Dr. Bergey, in some cases seemed to have been lost as regards certain carbohydrates, as I could not obtain fermentation with numerous tests and on different lots of the same kinds of media. No. 2, which was found by Dr. Bergey to belong to the group fermenting saccharose, dulcitol, adonitol, and inulin, seemed to have lost all power by the time my work was undertaken. No. 3 had lost the power to ferment lactose and sorbite; No. 8, the power to ferment

<sup>13</sup> Jour. Infect. Dis., 1916, 18, p. 358.

<sup>14</sup> Jour. Bacteriol., 1916, 1, p. 81.

\* Witte's peptone 0.5%, dextrose 0.5%, K<sub>2</sub>HPO<sub>4</sub> 0.5% are heated in 800 c.c. of water and stirred for 20 minutes; the whole is then filtered, cooled to 20 C., diluted to 1,000 c.c., and tubed. The medium is sterilized by the intermittent method.

TABLE 1  
FERMENTATIVE POWERS OF COLON ORGANISMS FROM VARIOUS SOURCES

Num- ber	Lac- tose	Sor- bite	Dex- trin	Dex- trose	Saccha- rose	Dul- cite	Man- nite	Mal- tose	Ado- nite	Inu- lin	Raffi- nose
FROM MILK											
1	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	+	—	—	+	—	—	—	—
4	—	—	—	—	—	—	—	—	—	—	—
5	+	+	—	+	—	—	+	+	—	—	—
6	—	—	+	+	+	—	—	+	—	—	—
7	+	+	—	—	+	+	+	+	—	—	+
8	+	—	—	—	—	—	—	—	—	—	—
9	+	+	+	—	—	—	+	—	—	—	+
10	+	—	+	+	+	—	+	+	—	—	+
11	+	+	+	+	+	—	+	+	—	—	+
12	+	+	—	+	—	—	+	+	—	—	—
13	+	+	—	+	+	+	+	+	—	—	+
14	+	+	+	+	+	+	+	+	—	—	+
15	—	—	—	—	—	—	—	—	—	—	—
16	+	—	—	+	+	—	+	—	—	—	—
17	+	+	—	+	—	—	+	+	+	—	—
18	+	+	—	+	+	+	+	+	—	—	+
FROM HUMAN FECES											
1	+	+	+	+	+	+	+	+	—	—	+
2	+	+	—	+	+	—	+	+	—	—	+
3	+	+	—	+	—	+	+	+	—	—	—
4	+	+	—	+	+	—	+	+	—	—	—
5	+	+	—	+	—	—	+	+	+	—	+
6	+	+	—	+	—	—	+	+	+	—	—
7	+	+	—	+	+	—	+	+	+	—	—
8	—	—	—	+	—	—	+	+	+	—	+
9	+	—	—	+	—	—	—	+	+	—	+
10	—	—	+	+	—	—	+	+	+	—	+
FROM RABBIT FECES											
1	+	+	—	+	+	—	+	+	—	—	+
2	+	+	+	+	+	+	+	+	—	—	+
FROM WATER											
1	+	+	—	+	+	—	+	+	+	—	+
2	+	+	—	+	+	—	+	+	—	+	—
3	+	+	+	+	+	—	+	+	—	+	—
4	—	—	—	—	—	—	—	—	—	—	—
FROM SEWAGE											
1	+	+	+	+	+	—	+	+	—	—	+
2	—	—	—	—	—	—	—	—	—	—	—
3	+	+	—	+	+	—	—	+	—	+	—
4	—	+	—	+	+	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—	—
FROM HUMAN URINE (PYELITIS)											
1	+	+	—	—	+	—	+	+	—	—	+
2	—	—	+	+	+	—	+	+	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—
FROM EGG POWDER											
1	+	—	+	+	+	—	+	—	—	—	—
2	+	+	+	—	+	+	+	+	+	—	+
3	—	+	—	+	+	—	—	+	—	—	—

TABLE 2

OTHER PROPERTIES OF COLON ORGANISMS FROM VARIOUS SOURCES

Num- ber	Voges-Proskauer Reaction	Liquefaction of Gelatin in Mm.	Indol- Production	P— Nitro- phenol	Methyl Red
FROM MILK					
1	+	3	—	Alkaline	Alkaline
2	+	0	—	Alkaline	Alkaline
3	+	40	+	Alkaline	Alkaline
4	+	5	—	Alkaline	Alkaline
5	—	5	—	Acid	Acid
6	+	40	+	Alkaline	Alkaline
7	+	0	+	Alkaline	Alkaline
8	+	0	+	Alkaline	Alkaline
9	+	0	+	Alkaline	Alkaline
10	+	0	+	Alkaline	Alkaline
11	+	0	+	Alkaline	Alkaline
12	—	0	+	Acid	Acid
13	+	0	—	Alkaline	Alkaline
14	+	15	+	Alkaline	Alkaline
15	+	3	+	Alkaline	Alkaline
16	—	10	—	Acid	Acid
17	—	0	—	Acid	Acid
18	—	0	+	Acid	Acid
FROM HUMAN FECES					
1	—	0	+	Acid	Acid
2	—	0	+	Acid	Acid
3	—	0	+	Acid	Acid
4	—	0	—	Acid	Acid
5	—	4	+	Acid	Acid
6	—	0	+	Acid	Acid
7	—	0	+	Acid	Acid
8	—	0	+	Acid	Acid
9	—	0	+	Acid	Acid
10	—	2	+	Acid	Acid
FROM RABBIT FECES					
1	—	0	+	Acid	Acid
2	—	0	+	Acid	Acid
FROM WATER					
1	—	10	+	Acid	Acid
2	—	20	—	Acid	Acid
3	+	8	—	Alkaline	Alkaline
4	—	4	+	Acid	Acid
FROM SEWAGE					
1	+	8	..	Alkaline	Alkaline
2	+	0	—	Alkaline	Alkaline
3	+	35	..	Alkaline	Alkaline
4	—	0	..	Acid	Acid
5	+	0	—	Alkaline	Alkaline
FROM HUMAN URINE					
1	—	0	+	Acid	Acid
2	—	13	—	Acid	Acid
3	+	0	—	Alkaline	Alkaline
FROM EGG POWDER					
1	+	0	+	Alkaline	Alkaline
2	+	0	+	Alkaline	Alkaline
3	+	0	—	Alkaline	Alkaline

TABLE 3  
CLASSIFICATION OF COLON BACILLI ACCORDING TO BERGEY AND DEEHAN

Source	S- D- A- I-	S+ D- A- I-	S- D+ A- I-	S- D- A+ I-	S- D- A- I+	S+ D+ A- I-	S+ D- A+ I-	S+ D- A- I+	S- D+ A+ I-	S- D+ A- I+	S- D+ A+ I+	S+ D+ A+ I-	S+ D+ A- I+	S+ D- A+ I+	S+ D+ A+ I+	S+ D+ A+ I+	Total
Milk.....	9	4	0	1	0	4	0	0	0	0	0	0	0	0	0	0	18
Human feces.....	0	2	1	5	0	1	1	0	0	0	0	0	0	0	0	0	10
Rabbit feces.....	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	2
Water.....	1	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	4
Sewage.....	2	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	5
Urine.....	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Egg powder.....	0	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	3
Total.....	13	13	1	6	0	6	2	3	0	0	0	0	1	0	0	0	45

S = Saccharose; D = Dulcitol; A = Adonitol; I = Inulin.

TABLE 4  
DISTRIBUTION OF COLON ORGANISMS AMONG MACCONKEY'S GROUPS

Source	B. Acidi- Lactici Saccharose — Dulcitol —	B. Coli- Communis Saccharose — Dulcitol +	B. Coli- Communior Saccharose + Dulcitol +	B. Lactis- Aerogenes Saccharose + Dulcitol —	Total
Milk.....	10	0	4	4	18
Human feces.....	5	1	1	3	10
Rabbit feces.....	0	0	1	1	2
Water.....	1	0	0	3	4
Sewage.....	3	0	0	2	5
Urine.....	1	0	0	2	3
Egg powder.....	3	0	1	2	3
Total.....	20	1	7	17	45

TABLE 5  
CORRELATION BETWEEN THE SOURCE OF COLON ORGANISMS AND SACCHAROSE-FERMENTATION

Source	Saccharose +		Saccharose —	
	Number of Organisms	Percentage	Number of Organisms	Percentage
Milk.....	8	44	10	56
Human feces.....	4	40	6	60
Rabbit feces.....	2	100	0	0
Water.....	3	75	1	25
Sewage.....	2	40	3	60
Urine.....	2	67	1	33
Egg powder.....	3	100	0	0

TABLE 6  
CORRELATION OF THE VOGES-PROSKAUER AND METHYL-RED REACTIONS WITH SOURCE

Source	Reaction to Methyl Red, 5 Days' Incubation		Voges-Proskauer Reaction	
	Acid or Neutral	Alkaline	Positive	Negative
Milk.....	5	13	13	5
Human feces.....	10	0	0	10
Rabbit feces.....	2	0	0	2
Water.....	3	1	1	3
Sewage.....	1	4	4	1
Urine.....	2	1	1	2
Egg powder.....	0	3	3	0

sorbite, raffinose, saccharose, maltose, adonite; Nos. 10 and 11, the power to ferment adonite and inulin; No. 12, the power to ferment adonite.

Of the organisms from human feces Nos. 1, 2, and 4 were varieties of *B. coli-communior*; No. 3 was a variety of *B. coli-verus*; Nos. 5 and 6 were varieties of *B. acidilactici*; No. 7 was *B. coli-aerogenes*; and Nos. 8, 9, and 10 were varieties of *B. dysenteriae*. Both the organisms from rabbit feces were varieties of *B. coli-communior*.

Of the water colon bacilli No. 3 had lost the power to ferment dulcitate and adonite; No. 4 the power to ferment lactose, raffinose, dextrin, mannite, and inulin. Of those from sewage, Nos. 2 and 5 were *alkaligenes* types.

The classification according to saccharose-fermentation alone shows a greater correlation with source than do classifications by means of saccharose and dulcitate.

*Correlation between Voges Proskauer and Methyl Red Reactions.*—The 22 cultures that were alkaline to methyl red gave the Voges-Proskauer reaction, while all the cultures that were acid to methyl red did not. From Table 6 it will be seen that a definite relation exists between the source and the Voges-Proskauer and methyl-red reactions. Of the milk cultures 13, or 72%, gave the Voges-Proskauer reaction and all of these were alkaline to methyl red; of the cultures from human feces none gave the Voges-Proskauer reaction and none were alkaline to methyl red; of the rabbit feces none gave the Voges-Proskauer reaction nor was any one alkaline to methyl red; of the water cultures only 1, or 25%, gave the Voges-Proskauer reaction and it was alkaline to methyl red, while of the sewage cultures 4, or 80%, gave the reaction and were alkaline to methyl red; of the cultures from egg powder all 3 gave the Voges-Proskauer reaction and were alkaline to methyl red; of the cultures from human urine only 1 gave this reaction and it was alkaline to methyl red. These findings agree with those of Clark and Lubs and Levine that methyl-red-negative organisms are probably of nonfecal origin.

#### SUMMARY

A study of 45 organisms isolated from milk, human feces, rabbit feces, water, sewage, urine, and egg powder shows that—

There is a better correlation between saccharose-fermentation and source than between saccharose-dulcitate-fermentation and source.

The methyl-red and Voges-Proskauer reactions are related, as suggested by Levine. All organisms alkaline to methyl red gave a positive Voges-Proskauer reaction, and all organisms acid to methyl red gave a negative Voges-Proskauer reaction.

Organisms that gave a positive Voges-Proskauer reaction were not found in feces and hence it seems that this and the methyl-red reactions are of sanitary importance in determining colon organisms of fecal origin.

# THE FATE OF THE MAMMALIAN TUBERCULOSIS BACILLUS IN SPARROWS AND CHICKENS\*

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In an experiment in which a number of common sparrows were fed with tuberculous material of mammalian origin, it was observed that after a time the animals would succumb in a markedly emaciated condition but almost entirely free from any gross lesions resembling tuberculosis.

The material used was derived from cavia which had died of tuberculosis and which had been used for the propagation in vivo of 3 strains of mammalian bacilli, 2 of bovine and 1 of human origin. In the experiment with sparrows the various strains were also kept strictly separate, and the birds received only one meal of tuberculous material. The sparrows, which had been kept in captivity for several weeks, completely consumed the material offered. The cages were then cleaned and disinfected.

In Series 1727 (bovine) a cavia succumbing to the eighth passage of bacilli was used for material; in Series 1747 (bovine) one dying as a result of the fourth passage of the bacilli was used, while in Series 1775 (human) a cavia dead with tuberculosis caused by a second passage of the virus, was used. In Series 1747 a number of sparrows were fed with material from a cavia injected with material from a sparrow of the first lot used in the experiment.

After the first few deaths, especially in view of the unusually marked loss of flesh in the sparrows, the question of a possible survival, if not propagation, of the mammalian bacilli introduced, presented itself. In order to find our answer to this question the organs of the dead sparrows, notably the livers, were carefully examined for bacilli, while material from the livers of a number of the birds, was injected intraperitoneally into guinea-pigs, and in a few instances also into ordinary barnyard fowls. In the latter case the injections were made intravenously.

Table 1 permits a review of the results obtained in these inoculation experiments.

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TABLE 1

RESULTS OF EXPERIMENTS TO PROVE THE SURVIVAL OR PROPAGATION OF TUBERCLE BACILLI

Sparrow	Died After Days	Lesions	Bacilli in Organs	Transmitted to Cavia	Result of Transmission	Remarks
SERIES 1727. USING MATERIAL FROM CAVIA C <sup>18</sup>						
2658	14	None	None			
2659	63	Small nodule in liver	Present			
2657	70	None	Present	2656 2655		Both caviae were lost
2654	93	None	None	2635 2626	Tuberculosis Tuberculosis	
2620	147	None	None	2622 2621	Negative Negative	
2623	148	None	None	2624 2625	Negative Negative	
SERIES 1747. USING MATERIAL FROM CAVIA B <sup>1</sup>						
2764	52	None				
2844	72	None	None	2831 2845	Tuberculosis Tuberculosis	
2874	74	None	None	2859 2858	Tuberculosis Tuberculosis	
2876	111	None	None	2878 2877	Negative Negative	
2875	122	None				
2824	20	None	None	2825 2826 2810	Negative Negative Negative	
2838	35	None	None	2842 2841 2840 2839	Negative Negative Negative Negative	Infectiousness of chickens was not checked
2833	39	None	None	2837 2836 2835 2834	Negative Negative Negative Negative?	Had some bacilli in liver
2832	190 Killed	None	None			
SERIES 1775. USING MATERIAL FROM CAVIA B <sup>2</sup>						
2745	40	None	None	2744 2743	Tuberculosis	
2731	129	None	Present	2730 2730a	Tuberculosis Negative	
2725	136	None	None	2724 2723	Negative Negative	
2722	157					Material lost
2721	141	None	None	2720 2719	Tuberculosis	
2706	187	None	None	2702 2703 2704 2705	Negative Negative Negative Negative	

In all there were fed thus 21 sparrows, which, with only one exception (Sparrow 2832), died in an emaciated condition. Only in Sparrow 2659 was there any suggestion of tuberculous disease. This consisted of a very small nodule in the liver and in this nodule typical tubercle bacilli could be demonstrated microscopically.

The fact that the sparrows possibly died as a result of the ingestion of tuberculous material of mammalian origin is not the most striking feature of the findings recorded in the preceding table. It is rather the more or less persistent survival of mammalian tubercle bacilli in the body in birds which attracts our attention. While not all animals in the series were examined with this point in view, the persistence of the bacilli was manifest in a sufficient number to arouse interest.

The organ examined, from which material for the inoculation of guinea-pigs was taken, was the liver in all cases. In but 3 cases (Sparrows 2659, 2657, and 2731) could the organism be demonstrated microscopically in such manner as to leave no room for doubt.

In 6 cases we succeeded in causing tuberculosis in caviae by the intraperitoneal injection of small amounts of emulsions of sparrow livers (Sparrows 2654, 2844, 2874, 2745, 2731, and 2721). The material was collected 93, 72, 74, 40, 129, and 141 days, respectively, after the ingestion of the infective meal, so that it appears the mammalian tubercle bacillus may maintain itself for long periods in the organs of a bird without losing its vitality or its original virulence.

These observations suggested further inquiry with a view to determining whether or not a similar survival of the mammalian tuberculosis bacillus takes place when it is introduced into the body of common barnyard fowls. If such were the case, it would be well within the range of possibility that the common chicken, through its omnivorous habits and intimate contact with farm animals, with their ejecta and offal, might become a carrier of mammalian tuberculosis.

In the subsequent experiments designed to demonstrate this possibility, use was made of the same tuberculous materials as were used in the previous trial of sparrows. Most of the material was from caviae which had become tuberculous as a result of injection with emulsions of sparrow livers. In one series we used material directly from sparrows that had been fed with other sparrow material, and in another series, material from sparrows fed with mammalian tuberculous matter.

In the investigation under consideration all the chickens were injected intravenously, and the control caviae intraperitoneally. In checking up the infectiveness of chicken material, emulsions of the liver were used, with the exception of a few instances when emulsions of the spleen were also used. In the selection of cavia material such parts were taken as most warranted the assumption that they contained tubercle bacilli.

Tables 2 and 3 permit a review of the details of these experiments.

TABLE 2

RESULTS OF EXPERIMENTS TO PROVE THE SURVIVAL OF TUBERCULOSIS BACILLI OF BOVINE ORIGIN IN CHICKENS. SERIES 1727

Chicken	Source of Infection	Died After Days	Lesions	Bacilli in Organs	Transmitted to	Results of Transmission
2638	Cavia 2635	36	None	+	Cavia 2639 Cavia 2640 Chicken 2642 Chicken 2641	None Tuberculosis
2647	Cavia 2635	113	None	+	Cavia 2651 Cavia 1650 Chicken 2653 Chicken 2652	Tuberculosis Tuberculosis
2642	Chicken 2638	30	None	None	Cavia 2646 Cavia 2645 Chicken 2644 Chicken 2644a	None None
2641	Chicken 2638	146	None	?	Cavia 2933 Cavia 2934 Chicken 2931 Chicken 2932	None None
2653	Chicken 2647	228 Killed	None	None	Cavia 3269 Cavia 3270	None None
2652	Chicken 2647	228 Killed	None	None	Cavia 3271 Cavia 3272	None None
2644	Chicken 2642	244 Killed	None	None	Cavia 3267 Cavia 3268	None None
2644a	Chicken 2642	246 Killed	None	None	Cavia 3277 Cavia 3278	None None
2931	Chicken 2641	179 Killed	None	None	Cavia 3306 Cavia 3307	None None
2932	Chicken 2641	148 Killed	None	None	Cavia 3239 Cavia 3240	None Tuberculosis
2629	Cavia 2626	326 Killed	None	None	Cavia 3246 Cavia 3247	None None
2630	Cavia 2626	19	None	+	Cavia 2633 Cavia 2624 Chicken 2632 Chicken 2631	Tuberculosis Tuberculosis
2632	Chicken 2630	323 Killed	None	None	Cavia 3279 Cavia 3280	None None
2631	Chicken 2630	296	None	None	Cavia 3214 Cavia 3215	None None

In the group recorded in Table 2 there were 4 chickens which received each an intravenous injection of an emulsion prepared from the lesions of a cavia that had become tuberculous as the result of inoculation with material from sparrows carrying tuberculosis bacilli in the liver. Of these, 3 (2638, 2647, and 2630) died 36, 113, and 19 days, respectively, after inoculation, and guinea-pigs injected with

TABLE 3

RESULTS OF FURTHER EXPERIMENTS TO PROVE THE SURVIVAL OF TUBERCULOSIS BACILLI OF BOVINE ORIGIN IN CHICKENS. SERIES 1747

Chicken	Source of Infection	Died After Days	Lesions	Bacilli in Organs	Transmitted to	Results of Transmission	Remarks
2847	Cavia 2845	89	None	+	Cavia 2851 Cavia 2850 Chicken 2849 Chicken 2848	Tuberculosis Tuberculosis	
2846	Cavia 2845	145	None	+			
2849	Chicken 2847	268 Killed	None	?	Cavia 3285 Cavia 3286	None None	
2848	Chicken 2847	177	None	None	Cavia 3092 Cavia 3093 Chicken 3094 Chicken 3095	None	
3094	Chicken 2848	111 Killed	None	None	Cavia 3308 Cavia 3309		Cavias died of sepsis
3095	Chicken 2848	85	None	None	Cavia 3261 Cavia 3262	None Tuberculosis	
2869	Cavia 2869	89	None	None	Cavia 2870 Cavia 2871 Chicken 2872 Chicken 2873	None	
2867	Cavia 2859	358 Killed	None	None	Cavia 3248 Cavia 3249	None None	
2860	Cavia 2859	120	?	?	Cavia 2866 Cavia 2865 Chicken 2862 Chicken 2861	None None	
2872	Chicken 2869	265 Killed	None	None	Cavia 3237 Cavia 3238	None None	
2873	Chicken 2869	81	None	?	Cavia 2892 Cavia 2893 Chicken 2894 Chicken 2895		Cavias died of sepsis
2862	Chicken 2860	18	None	+	Cavia 2864 Cavia 2863	None None	
2861	Chicken 2860	93	None	None	Cavia 2949 Cavia 2950 Chicken 2948 Chicken 2947	None None	
2894 2895							Material lost through sepsis
2948	Chicken 2861	46	None	None	Cavia 3049 Cavia 3050 Chicken 3047 Chicken 3048		Sepsis
2947	Chicken 2861	20	None	None			Injected animals died with sepsis
3047	Chicken 2948	22	None	None	Cavia 3106 Cavia 3107	None None	Cavias died with sepsis

TABLE 3—Continued

RESULTS OF FURTHER EXPERIMENTS TO PROVE THE SURVIVAL OF TUBERCULOSIS BACILLI OF BOVINE ORIGIN IN CHICKENS. SERIES 1747

Chicken	Source of Infection	Died After Days	Lesions	Bacilli in Organs	Transmitted to	Results of Transmission	Remarks
3048 2856	Chicken 2948 Cavia 2858	7 183	None None	None None	Cavia 2945 Cavia 2946 Chicken 2943 Chicken 2944	None None	Sepsis Cavias died with sepsis
2857	Cavia 2858	328 Killed	None	None	Cavia 3250 Cavia 3251	None None	
2943	Chicken 2856	173 Killed	None	None	Cavia 3310 Cavia 3311	None None	
2944	Chicken 2856	172 Killed	None	None	Cavia 3304 Cavia 3305	None None	
2826	Sparrow 2824	75	None	None	Cavia 2830 Cavia 2829 Chicken 2828 Chicken 2827	None None	
2810	Sparrow 2824	43	?	None	Cavia 2822 Cavia 2823 Chicken 2817 Chicken 2811	None None	
2828	Chicken 2826	88	None	None			
2827	Chicken 2826	93	None	None			
2817	Chicken 2810	59	None	None	Cavia 2821 Cavia 2820 Chicken 2818 Chicken 2819	None None	
2811	Chicken 2810	83	None	None	Cavia 2816 Cavia 2815 Chicken 2814 Chicken 2813 Chicken 2812	None None	
2818	Chicken 2817	76 Killed	Tuber- culosis	+			
2819	Chicken 2817	76 Killed	None				
2814	Chicken 2811	24	None	+			
2813	Chicken 2811	39	None				
2812	Chicken 2811	43	None				
2840	Sparrow 2838	182 Killed	None				
2839	Sparrow 2838	183 Killed	None				
2835	Sparrow 2833	149 Killed	None				
2834	Sparrow 2833	149 Killed	Tuber- culosis	+			

material from their livers developed tuberculosis in due course of time. In all three cases the bacillus could be demonstrated in liver smears. The fourth (Chicken 2629) was killed 326 days after inoculation, and both by microscopical examination and by injection into a cavia was proved to be free from infection.

Material from the three chickens which proved to be infective for guinea-pigs was injected into 6 other chickens, with the result that 3 (2642, 2641, and 2630) died 30, 146, and 296 days after the injection; the remaining three (2653, 2652, and 2632) were killed after periods of observation of 228, 228, and 323 days respectively. In none was it possible to show the existence of tuberculous infection by direct examination or by transmission to caviae, altho in the liver of Chicken 2641 some acidfast debris suggestive of the tuberculosis bacillus was found.

From 2 of the six, material was injected into 4 other chickens (2644, 2644a, 2931, and 2932). None died spontaneously. They were killed after 244, 246, 179 and 148 days, and material from the livers injected into guinea-pigs proved noninfective, with the exception of that taken from Chicken 2932, which produced tuberculosis in one of 2 caviae injected.

In none of the chickens used in Series 1727 could any lesions suggestive of tuberculosis be observed, but all the birds dying spontaneously showed a most marked state of emaciation.

In the trials made with materials of Series 1747, we were unfortunate in losing several caviae, either on account of an apparently unavoidable acute septic infection or as a result of certain toxic materials associated with the chicken material used.

Seven chickens (2874, 2846, 2869, 2867, 2860, 2856, and 2857) were injected with virulent cavia material. Of this number 5 (2847, 2846, 2869, 2860, and 2856) died 89, 145, 89, 120, and 183 days, respectively, after the injection, while 2 (2867 and 2857) were killed while in apparently good health after 358 and 328 days.

Of the number injected only 2 showed the tuberculosis bacillus. In Chicken 2847 this was demonstrated both by inoculation and direct examination, while in the other the result of the microscopical examination of material taken from the liver left no doubt in regard to the presence of the organism mentioned.

Only in Chicken 2860 of this group of seven could anything resembling a tuberculous lesion be detected at autopsy. This lesion consisted of a questionable nodule in the liver, in which also some acidfast debris was found on microscopical examination. The caviae injected with an emulsion of this material, however, escaped infection.

Two chickens were injected with material from Chicken 2847, the one which had proved infective to cavia. One, 2848, died 177 days after inoculation and material taken from it and inoculated into guinea-pigs failed to produce the disease. The other (Chicken 2849) which was killed after 268 days, also proved to be free from tuberculosis infection, altho in its liver some questionable acidfast debris was found.

From certain of the noninfective members of the group of seven, transfers were made to 6 other fowls. Three of the latter (2873, 2862, and 2861) died 81, 18, and 93 days after inoculation, while the remaining three (2872, 2943, and 2944) were killed in apparently good health 265, 173, and 172 days after inoculation. Neither from the birds which died, nor from the ones that were killed, could tuberculosis be transmitted to cavia. In one of the former (2873) questionable organisms were found in the liver and in another (2862) tuberculosis bacilli could be demonstrated in the same organ. As the cavia escaped infection, it must be assumed that the bacilli found were dead. In Chicken 2873 it was impossible definitely to prove or disprove the infectiousness of the material because of the loss of the cavia from sepsis.

From Chicken 2848 injected with material which had proved infectious to cavia and which this bird apparently failed to retain in virulent form, material was injected into 2 other chickens (3094 and 3095). One died 85 days later, and the other was killed after 111 days. The latter's infectiveness could not be established, the cavia injected being lost through sepsis, but 1 of the 2 guinea-pigs injected with material from the former developed the disease in an unmistakable manner.

Of 2 more chickens (2948 and 2947) inoculated with material taken from one of the group of 5 birds mentioned (2861) the infectiousness could not be ascertained because of the loss of the cavia used. Both died after 46 and 20 days.

Six chickens in Series 1747 were inoculated with sparrow material. The sparrows (2824, 2838, and 2833) had been fed with tuberculous guinea-pig organs, but material taken from them after death was not infective for cavia.

Two of the six (2826 and 2810) died after 75 and 43 days. The remaining four (2840, 2839, 2835, and 2834) were killed 183, 183, 149 and 149 days after inoculation. In one (2810) some nodules were found in the liver, but the presence of the tuberculosis bacillus could

not be demonstrated either by microscopical examination or by animal inoculation. In another of this group (2834) 3 small nodules were found in the liver, containing typical tubercle bacilli in large numbers.

From the two which died spontaneously (2826 and 2810) material was injected into 4 healthy chickens (2828, 2827, 2817, and 2811). All died spontaneously 88, 93, 59, and 83 days after the injections. None showed lesions or other evidence of infection.

From 2 of the four chickens a group of 5 other fowls were injected. Two of these (2818 and 2819) were killed after 76 days, one of which showed lesions and bacilli in the liver. The remaining three (2814, 2813, and 2812) died 24, 39, and 43 days after the inoculation. None showed lesions, but one (2814) had some bacilli in the liver.

From a sparrow in this series (2706) which after being fed with tuberculous material had failed to carry infective material until its death, material was injected into 2 chickens (2704 and 2705). The former died after 215 days, and the latter was killed while in good health 308 days after the inoculation. These two birds were free of lesions, and bacilli could not be demonstrated either by animal inoculation or by direct microscopical examination. Two chickens (3068 and 3069), injected with liver emulsion from the one chicken which died spontaneously, and killed 134 and 142 days afterward, also were free from lesions and bacilli.

Seven chickens in this series were injected with infective cavia material. Five (2742, 2732, 2737, 2729, and 2715) died 112, 295, 125, 137, and 23 days, respectively, after the inoculation, while the other two (2728 and 2716) were killed in normal condition after 150 and 331 days. Of this entire group only one animal (2715) contained bacilli in the liver.

From the seven chickens liver emulsions were injected into 6 normal fowls (2740, 2741, 2735, 2736, 2712, and 2711). Three (2740, 2741, and 2711) died spontaneously after 243, 159, and 74 days; the remaining three (2735, 2736, and 2712) were killed while in good health after 246, 230, and 296 days. In only one of the six (2711) could bacilli be demonstrated.

Of this last group, 2 fowls (2741 and 2711) furnished material for further inoculations into 4 other chickens; of these only one died spontaneously (2709), after 163 days; the other three (3064, 3065, and 2710) were killed while in good health, after 129, 121, and 222 days. In none of the four could tuberculous infection be demonstrated.

TABLE 4

RESULTS OF EXPERIMENTS TO PROVE THE SURVIVAL AND PROPAGATION OF TUBERCLE BACILLI  
OF HUMAN ORIGIN IN CHICKENS. SERIES 1775

Chicken	Source of Infection	Died After Days	Lesions	Bacilli in Organs	Transmitted to	Results of Transmis- sion	Remarks
2704	Sparrow 2706	215	None	None	Cavia 3066 Cavia 3067 Chicken 3068 Chicken 3069	None None	
2705	Sparrow 2706	308 Killed	None	None	Cavia 3257 Cavia 3258	None None	
3068	Chicken 2704	134 Killed	None	None	Cavia 3314 Cavia 3315	None None	
3069	Chicken 2704	142 Killed	None	None	Cavia 3338 Cavia 3339	None None	
2742	Cavia 2743	112	None	None	Cavia 2738 Cavia 2739 Chicken 2740 Chicken 2741	None None	
2732	Cavia 2743	295			Cavia 3137 Cavia 3138	None None	
2737	Cavia 2743	125	None	None	Cavia 2733 Cavia 2734 Chicken 2735 Chicken 2736	None None	
2740	Chicken 2742	243	None	None	Cavia 3236 Cavia 3235	None None	
2741	Chicken 2742	159	None	None	Cavia 3062 Cavia 3063 Chicken 3064 Chicken 3065	None None	
2735	Chicken 2737	346 Killed	None	None	Cavia 3283 Cavia 3284	None None	
2736	Chicken 2737	230 Killed	None	None	Cavia 3229 Cavia 3230	None None	
3064	Chicken 2741	129 Killed	None		Cavia 3340 Cavia 3341	None None	
3065	Chicken 2741	121 Killed	None	None	Cavia 3312 Cavia 3313	None None	
2728	Cavia 2730	150	None	None	Cavia 3121	None	
2729	Cavia 2730	Killed 137			Cavia 3122	None	Lost
2716	Cavia 2719	331 Killed	None	None	Cavia 3281 Cavia 3282	None None	
2715	Cavia 2719	23	None	+	Cavia 2714 Cavia 2713 Chicken 2712 Chicken 2711	Tuberculosis Tuberculosis	
2712	Chicken 2715	296 Killed	None	None	Cavia 3241 Cavia 3442	None None	
2711	Chicken 2715	71	None	+	Cavia 2707 Cavia 2708 Chicken 2709 Chicken 2710	Tuberculosis None	
2709	Chicken 2711	163	None	None			
2710	Chicken 2711	222 Killed	None	None	Cavia 3265 Cavia 3266	None None	

TABLE 5  
RESULTS OF FEEDING EXPERIMENTS WITH TUBERCLE BACILLI

Chicken	Source and Date of Infection	Lesions	Results of Feces Examination				Results of Liver Examination			
			Date	Bacilli in Smears	Cavia Inoculation		Date	Bacilli in Smears	Cavia Inoculation	
					Cavia	Results			Cavia	Results
2937	Cavias, Series 1775,* 1/22/15	None	2/ 8/15	?	2973	Negative	7/22/15	None	3327 3328	Negative Negative
			2/22/15	None	2974	Negative				
					3000	?				
					3001	?				
			3/ 8/15	None	3030	Negative				
					3031	Negative				
			4/ 6/15		3112	Negative				
					3113	Negative				
			5/18/15	None	3186	Negative				
			7/22/15	None	3187	Negative				
2938	Spleen, Series 2919 (hog), 1/22/15	None	2/ 8/15	Acid fast debris	2975	Negative	7/22/15	None	3325 3326	Negative Negative
			2/22/15	None	2976	Negative				
					3004	Negative				
					3005	Negative				
			3/ 8/15	Acid fast debris	3032	Doubtful				
					3033	Doubtful				
			4/ 6/15		3114	Negative				
					3115	Negative				
			5/18/15	Acid fast debris	3188	Negative				
			7/22/15	None	3189	Negative				
2935	Cavias, Series 1727, 1/22/15		2/ 8/15	None	2969	Negative	7/22/15	None	3331 3332	Negative Negative
			2/22/15	None	2970	Negative				
					3002	Negative				
					3003	Negative				
			3/ 8/15	Acid fast debris	3026	Negative				
					3027	Negative				
			4/ 6/15		3108	Negative				
					3109	Negative				
			5/18/15	Present	3182	Negative				
			7/22/15	None	3183	Negative				
2936	Cavias, Series 1747, 1/22/15		2/ 8/15	Present	2971	Positive	7/22/15	None	3329 3330	Negative Negative
			2/22/15	None	2972	Negative				
					2998	Negative				
					2999	Negative				
			3/ 8/15	None	3028	Negative				
					3029	Positive				
			4/ 6/15		3110	Negative				
					3111	Negative				
			5/18/15	None	3184	Negative				
			7/22/15	None	3185	Negative				
					3320	Negative	7/22/15	None	3329 3330	Negative Negative
					3121	Negative				

\* First five cavias died of sepsis.

In addition to the trials described, some further experiments were made in which chickens were given one meal of infectious material and in which the feces were examined from time to time for the presence of bacilli. The chickens in this experiment were permitted to live for 6 months, after which they were killed so that the liver could be examined for bacilli. While, unfortunately, lack of room limited

the number of animals used and this small number does not permit definite conclusions, it seems well to make mention of this trial in connection with the work already described.

The material taken from caviars belonging to Series 1727, 1747, and 1775 was fed to 3 chickens, while that of Series 2919 from a tuberculous hog spleen was given to a fourth fowl. Table 5 permits a review of this experiment.

From Table 5 it is apparent that in only one of the four chickens fed with tuberculous material, was it possible to show that the feces contained virulent bacilli for some time after the material was fed. This was Chicken 2936, the ejecta of which proved virulent 17 and 45 days after the infective meal. In one (2938) the results were doubtful when feces passed after 45 days were examined. In the other two fowls (2935 and 2937) the results were negative.

At the time the fowls were killed, they were apparently in good health, altho one of them had lost considerable flesh during the first month after the feeding. This chicken, however, recovered and showed no further evidence of illness.

In a number of caviars, representing all the series, some doubtful nodular formations were encountered in certain organs, but in none could the existence of tuberculosis be definitely shown.

Summarizing the results obtained we find as follows:

{ Sparrows fed with virulent mammalian material and examined.....	18
{ Sparrows found to contain tubercle bacilli after from 40 to 141 days....	8
{ Chickens injected with virulent mammalian material and examined.....	17
{ Chickens found to contain tubercle bacilli after from 19 to 145 days.....	6
{ Chickens injected with material from sparrows fed with virulent mam-	
{   malian matter .....	8
{ Chickens found to contain tubercle bacilli after 149 days.....	1
{ Chickens fed with virulent mammalian material.....	4
{ Chickens the feces of which contained virulent bacilli after 45 days.....	1

In addition to the groups of chickens summarized, there were injected with material from them 25 other fowls, of which only 2 proved to be infective after 18 and 74 days.

From some of these twenty-five chickens material was injected into 17 healthy fowls, in 4 of which tubercle bacilli were demonstrated 24, 76, 85, and 148 days, respectively, after the inoculation.

An interesting feature of our experiments is that in a series of transmissions there was one or more animals in which no infection could be shown and yet animals inoculated with material furnished by them again were proved to contain tubercle bacilli, either by microscopical observation or by positive results in caviae. Table 6 shows the history of these transmissions and their results.

Table 6

Original Tuberculous Material	Transmission to	Results	Transmission to	Results	Transmission to	Results	Transmission to	Results	Transmission to	Results
Cavia, Series 1747	Sparrow 2833	Negative	Chicken 2834	Positive						
Cavia, Series 1747	Sparrow 2834	Negative	Chicken 2810	Negative	Chicken 2811	Negative	Chicken 2814	Positive		
Cavia, Series 1747	Sparrow 2824	Negative	Chicken 2810	Negative	Chicken 2817	Negative	Chicken 2818	Positive		
Cavia, 2870	Chicken 2860	Negative	Chicken 2862	Positive						
Cavia, 2845	Chicken 2847	Positive	Chicken 2848	Negative	Chicken 3025	Positive				
Cavia, Series 1727	Sparrow 2654	Positive	Cavia 2655	Positive	Chicken 2638	Positive	Chicken 2641	Negative	Chicken 2632	Positive

We have no definite data to indicate precisely the cause of this phenomenon and merely suspect it to be due to the smallness of the number of bacilli which survived in the body of the birds and which caused them to be missed either in the microscopical preparations or in the emulsions injected.

In regard to the mortality among birds which either ingested or were injected with mammalian material, the results may be summarized as indicated in Table 7.

TABLE 7  
SUMMARY OF RESULTS AS REGARDS MORTALITY

Animals	Source of Material Injected or Fed	Died	Killed	Remarks
Sparrows.....	Caviae.....	17		
Sparrows.....	Sparrows.....	3	1	
Chickens.....	Caviae.....	14	9	
Chickens.....	Sparrows.....	3	5	
Chickens.....	Chickens.....	23	22	Including all groups

Practically all birds which died after inoculation became greatly emaciated near the end, while all those which were killed at a considerable period after inoculation were without exception in a perfect state of health and nutrition.

#### CONCLUSIONS

A considerable number of birds into which mammalian tuberculosis bacilli are introduced, either by ingestion or by inoculation, die in a highly emaciated state.

As a result of the incorporation of such bacilli into the bodies of birds, the latter may retain the organisms for long periods with their pathogenic characteristics fully preserved.

In consequence it is well within the range of possibility that birds may serve as intermediary carriers and transmitters of mammalian tuberculosis.\*

\* Our results confirm, at least in part, certain observations made by Auclair in pigeons (*Arch. méd. exper. et d'anat. path.*, 1897, 9, p. 277). Auclair observed that 3 pigeons injected intraperitoneally with pure cultures of human bacilli died after from 1 to 3½ months, without showing any sign of tuberculosis. In a second series, pigeons were infected in a similar manner with tubercle bacilli from human sources. At the 6th, 7th and 14th day afterward, the pigeons were killed and the livers, lungs, and blood injected into cavia. A few of the latter died without any evidence of tuberculosis. Only 2 died of local tuberculosis.

Auclair concludes (1) that pigeons infected with human bacilli die without any tuberculous changes; (2) that tubercle bacilli may retain their vitality and virulence in the body of the pigeon for at least 14 days; (3) that tubercle bacilli localize themselves in the pigeon by preference in the liver and the lungs, but not, so far as could be shown, in the blood; and (4) that the tubercle bacilli passed through the pigeon give rise in the cavia to a slowly developing local tuberculosis.

# EXPERIMENTAL EPIDEMIOLOGY IN TUBERCULOSIS \*

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The keen interest shown in the study of epidemiology since medicine realized the idea of the infectivity of some diseases, demonstrates the importance of those studies from theoretic as well as from practical points of view. Particularly in tuberculosis that study has been carried on with much intensity. The enormous quantity of literature accumulated on the subject, its exhaustive treatment in so many excellent books, leads to a postulate, namely: The longer a community of people has been in contact with tuberculosis virus the less mortality is to be found among the persons constituting that community, tho almost 90% of civilized people in postmortem examination present healed tubercular lesions.

If we ask the cause of this diminishing mortality, we may inquire (1) whether the virus has become attenuated through passages, or (2) whether the persons concerned have acquired a resistance to the virus. These questions have been left without answer, and quite naturally, because observations or experiments on human beings are based on insufficient determinism, the subjects are never comparable, and we are unable to ascertain how far diseases are antagonistic to each other. It would be of great interest to put these problems on an experimental footing, and thereby ascertain the biologic laws which govern those established postulates; in other words, we could inquire how tuberculous process is spread, what are the probable channels of infection, and what are the conditions under which resistance or immunity is established. Those were the problems in my mind, and I give in this work the first results of my investigations.

The experiments carried out were extremely simple. Guinea-pigs were injected with a known dose of tubercle bacilli, and then fresh guinea-pigs coming straight from the caviary were put into the same cages at fixed intervals. No special precautions were taken as regards cleanliness. Small and large cages were cleaned every 7 days. The large cages were 60 cu. in. square, and the small cages measured 30 cu. in.

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## EXPERIMENTS

- 1.—A. 11/27/1914. Four guinea-pigs were injected subcutaneously with 0.1 mg. of tubercle bacilli each.
- 12/14. One guinea-pig found dead with generalized tuberculosis.
- B. 12/14. Four guinea-pigs arriving straight from the caviary were put in the same cage.
- A. 12/20. One found dead with tuberculous lesions in the liver and the spleen.
- B. 12/20. Two dead. Lesions in the spleen and the liver.
- C. Emulsion of the spleens of these two guinea-pigs injected into 2 fresh guinea-pigs.
- B. 12/21. Another guinea-pig dead with generalized tuberculosis.
- D. Emulsion of the spleen of the animal in B injected into 2 fresh guinea-pigs.
- C. 12/29. Found dead with very pronounced lesions.
- D. 1/ 4/1915. Killed. Generalized tuberculosis.
- B. 1/29. The last guinea-pig of B killed. Only small lesions in the spleen. Weight of animal normal.

It was decided to kill B4 because of its comparatively long life. At any rate, some doubt may exist as to whether the disease was retrogressing, beginning, or stopping; but the normal weight of this animal and its long life indicate that probably there was a slight tuberculous process from the beginning which had been mastered by the animal. Some of the guinea-pigs of Experiment A were living on Feb. 15, 1915. They were then employed for other experiments.

The injection of the emulsion of the spleen of the dead animals as mentioned gave positive results. In the following experiments only sections were made to confirm the microscopic lesions. From this experiment it is seen that of the fresh guinea-pigs put in contact with tubercular guinea-pigs, after 17 days of infection, 75% die with the utmost rapidity and exhibit tuberculous lesions on postmortem examination.

- 2.—A. 11/27/1914. Four guinea-pigs injected subcutaneously with 0.05 mg. of tubercle bacilli (half the dose used in Experiment 1).
- A. 12/ 5. One guinea-pig found dead. Small lesions in the spleen.
- B. 12/14. Four fresh guinea-pigs put into this cage.
- B. 12/15. One found dead. No lesions.
- B. 12/17. Another guinea-pig dead. Lesions in the spleen, and in the lung.
- B. 12/21. Another guinea-pig dead. Lesions in the spleen and in the liver.
- B. 1/12/1915. The last dead. Generalized tuberculosis and mesenteric ganglion. The spleen not enlarged.
- A. Three still living on March 10, 1915, when they were employed for other experiments.

This experiment confirms the first, and shows that the amount of the initial dose of tubercle bacilli does not materially affect infection by contact, and that infection by contact in 100% of the cases is lethal. Contacts die in less time than injected guinea-pigs.

- 3.—A. 11/27/1914. Four guinea-pigs injected subcutaneously with 0.1 mg. of tubercle bacilli.  
 B. 12/30. Two fresh guinea-pigs put in as contacts.  
 B. 1/25/1915. One found dead. Generalized tuberculosis and mesenteric ganglion.  
 B. 2/1. The other killed. Lesions in liver, lungs, and spleen. Spleen not enlarged. Large mesenteric ganglion without pus.

This experiment confirms the first two. The conditions of infection are still active after 1 month and 3 days.

- 4.—A. 12/17/1914. Six guinea-pigs injected with 1 mg. of tubercle bacilli.  
 B. 12/30. Two fresh guinea-pigs put into the same cage.  
 B. 1/28/1915. One dead. Generalized lesions. Mesenteric ganglion.  
 B. 2/1. The other killed. Only mesenteric ganglion.

It seems from this experiment that the virus is infectious 13 days after the infection, at least in 50% of the cases.

In B 2 as in B 4 of the first experiment, the weight was normal, and the lesions small. The guinea-pigs left in all these experiments were killed because it was necessary to determine first of all whether every contact was infected.

The questions arising in connection with these slightly infected animals are a subject for further inquiries.

- 5.—A. 3/27/1915. Four guinea-pigs injected subcutaneously with 1 mg. of tubercle bacilli.  
 4/28. One guinea-pig dead with generalized tuberculosis.  
 B. 4/28. Two newly arrived guinea-pigs put into the cage.  
 A. 5/3. Another guinea-pig found dead with lesion in the spleen.  
 A. 5/10. Another dead, with generalized tuberculosis.  
 6/8. The guinea-pigs left were killed. A—generalized tuberculosis; B—only small lesions in the lungs.

These experiments demonstrate that the virus is infectious from the 13th to the 33rd day. It was next desirable to inquire at what time the virus commences to be infective for contacts, and also when the virus becomes no longer infective.

- 6.—A. 5/27/1915. Eight guinea-pigs injected subcutaneously with 1 mg. of tubercle bacilli.  
 B. 6/17. Two contacts.  
 7/8. Killed because they were losing weight. Lesions in the lungs of one very evident. One part of the lungs of the other and the spleen affected.

This also confirms the previous experiment; the virus is still infective after 21 days from the infection.

- 7.—A. 4/22/1915. Six guinea-pigs injected with 0.5 mg. of tubercle bacilli.  
 B. 5/18. Three fresh guinea-pigs for contact.  
 A. 6/25. Dead.  
 B. 7/8. Killed. No lesions.

This experiment shows how difficult it is in biology to group observations in accordance with a rigid law. A certain amount of amplitude must be allowed for the experiments on account of virulence of the strains of tubercle bacilli and on account of individual factors, etc.

- 8.—A. 5/21/1915. Four guinea-pigs injected with 0.5 mg. of tubercle bacilli.  
 B. 6/9. Two fresh guinea-pigs for contact.  
 A. 7/2. Dead.  
 B. 7/8. Killed. No lesions.

This experiment follows No. 7, but in the majority of the experiments it can be said that an infection arises through contact. We therefore proceeded to inquire when the virus commences to be infectious, and when it ceases to be so.

- 9.—A. 7/17/1915. Four guinea-pigs injected with 1 mg. of tubercle bacilli.  
 B. 7/23.  
 C. 7/26.  
 D. 7/29.  
 E. 8/10.  
 F. 8/20.  
 A. 8/23. } Two healthy guinea-pigs arrived from the caviary as contacts.  
 E. 8/18. }  
 D. 8/24. } All dead.  
 8/24. } Both dead with pulmonary tuberculosis.  
 One dead. No lesions except cachexia.  
 All the injected animals being dead, I resolved to kill the contacts.

The results were as follows: B—no lesions; C—(1) lesions in the spleen, (2) no lesions; D—the guinea-pig left had lesions in the lungs; F—no macroscopic lesions.

From these experiments it seems that the virus is absolutely infectious from the 9th to the 24th day after injection.

- 10.—A. 7/17/1915. Four guinea-pigs injected with 1 mg. of tubercle bacilli.  
 B. 7/20.  
 C. 7/23.  
 D. 8/10.  
 E. 8/20.  
 A. 8/30. } Two healthy animals introduced into this cage.  
 B. }  
 C. } All dead.  
 D. }  
 E. }  
 B. 8/31. Killed.  
 C. 8/31. Killed.  
 D. 8/31. Killed.  
 E. 8/31. Killed.  
 B. 8/31. Negative.  
 C. 8/31. Negative.  
 D. 8/31. One showed lesions in the spleen, the other had lungs affected.  
 E. 8/31. One had lungs affected, the other revealed no lesions.

This experiment seems to confirm No. 9. There is a cycle in the infectivity of the tuberculous virus which is nil at the beginning, and which then increases its infectivity with the multiplication of the tubercle bacilli in the body of the infected animal. Now the question arises whether the virus is infective at the beginning and at the end of the process.

- 11.—A. 6/ 9/1915. Six guinea-pigs injected each with 1 mg. of tubercle bacilli.  
 B. 6/ 9. Two healthy animals introduced into this cage.  
 A. 7/ 8. One dead with generalized tuberculosis.  
 B. 7/ 8. Killed. No lesions.  
 C. 7/14. Two fresh contacts.  
 C. 8/31. Killed. No lesions.

This experiment shows (1) that fresh guinea-pigs put in contact with tuberculous guinea-pigs on the first day of the injection do not become tuberculous, and (2) that after 1 month and 22 days, the virus ceases to be infectious.

- 12.—A. 8/21/1915. Six guinea-pigs injected with 1 mg. of tubercle bacilli.  
 B. 8/21. Three guinea-pigs for contact were put into the same cage.  
 B. 8/ 3. One of the contacts was lost.  
 A. 10/ 4. All dead with tuberculous lesions.  
 B. 10/ 4. One found dead. No tubercular lesions; lungs full of gas.  
 B. 10/ 4. The contact left was killed. No lesions.

These experiments confirm No. 11.

- 13.—A. 1/29/1915. Three guinea-pigs injected with 1 mg. of tubercle bacilli.  
 B. 1/29. Three guinea-pigs for contact.  
 A. 3/18. Dead with generalized tuberculosis.  
 B. 4/20. No signs of diminution of weight, therefore animals tried for immunity (see No. 16).  
 14.—A. 1/29/1915. Three guinea-pigs injected subcutaneously with 1 mg. of tubercle bacilli each.  
 B. 1/29. Three fresh guinea-pigs for contact.  
 A. 3/18. One dead with generalized tuberculosis.  
 B. 4/20. No signs of diminution of weight. Tried for immunity (see No. 16).  
 15.—A. 1/20/1915. Four guinea-pigs injected with 1 mg. of tubercle bacilli each subcutaneously.  
 B. 1/20. Two guinea-pigs for contact.  
 B. 2/ 9. One found dead. No apparent tuberculous lesions. The small intestine as in diarrhea.  
 B. 4/20. The other guinea-pig showed no diminution of weight, and therefore was used for trial of immunity (see No. 16).

The following experiment was undertaken to ascertain whether guinea-pigs living for a long time in contact with tuberculous guinea-pigs acquire a state of immunity.

- 16.—
- |            |   |   |
|------------|---|---|
| 4/20/1915. | { | A1 from Experiment 15, injected with 0.1 mg. of living tubercle bacilli.          |
|            |   | A2 from Experiment 14, not injected.  |
|            |   | A3 from Experiment 14, injected as before.  |
|            |   | A4 from Experiment 14, injected as before.  |
|            |   | A5 from Experiment 13, not injected.  |
|            |   | A6 from Experiment 13, injected as before.  |
|            |   | A7 from Experiment 13, injected as before.  |
|            |   | B. Two guinea-pig controls injected each with 0.1 mg. of living tubercle bacilli. |
- 5/ 4. A1 found dead, cachectic. No macroscopic lesions were observed.
- 5/15. B1 found dead. One spot in the lungs; very cachectic.
- 5/18. A3 found dead. Much emaciated; a small ganglion under the sternum, without pus.
- 6/18. A4 found dead. Generalized tuberculosis. Pancreas also affected.
- 7/ 2. The other four and the controls killed. A2—no trace of lesions; A5—no trace of lesions; A7—generalized tuberculosis; and B2—generalized tuberculosis.

This experiment confirms the last one on the one hand and shows that for a demonstration of immunity subcutaneous inoculation is too severe a test. We hope to return to this matter later on.

The following experiments were made to ascertain whether after a considerable interval of time, the virus of tuberculosis is still infectious.

- 17.—A. 2/ 5/1915. Four guinea-pigs injected with 0.1 mg. of tubercle bacilli.
- B. 4/20. A fresh guinea-pig is put in.
- B. 6/ 9. Killed. No lesions.

The result of this experiment indicates that the older the disease, the less dangerous it is for the contacts. In fact, after 1 month and 15 days, the virus is no longer infectious.

- 18.—A. 5/27/1915. Eight guinea-pigs injected with 1 mg. of tubercle bacilli.
- B. 7/14. Two fresh guinea-pigs for contact just arrived.
- B. 8/31. Killed. No lesions at all in either.
- 19.—A. 5/21/1915. Four guinea-pigs injected with tubercle bacilli.
- B. 7/14. Two contacts arrived from the caviary.
- B. 8/31. Killed. No lesions in either.

It seems from these last experiments that a cycle of the infection through contact exists. There is a curve which is nil at the beginning, which rises to its highest point between the 9th and the 33rd days, and which then goes down again. As we have pointed out before, these experiments indicate that there is a biologic law, with wide variations due to the influence of environment, etc., which governs the results.

The next set of experiments was undertaken to ascertain the relationship between mother guinea-pigs and their young. It is regretted

that the following experiments are few in number, because the breeding went wrong.

- 20.—3/12/1915. A mother guinea-pig was injected with 1 mg. of tubercle bacilli. The 3 young, which were 4 days old, were left in her cage.
- 3/15. One of the young dead. No lesions. Von Pirquet test on the two left.
- 4/19. Both dead. No lesions. The reaction was negative. The mother living, and used for other experiments.

It has frequently occurred to me that the young die for very little cause. In this case the shock of the carrying out of the von Pirquet test probably caused the death of the young guinea-pigs.

- 21.—3/12/15. A mother guinea-pig injected with 1 mg. of tubercle bacilli. Three young, which were 4 days old when the mother was injected, were left in her cage.
- 4/12. Mother dead. Lesions in lungs. Very cachectic.
- 4/27. One young dead with no lesions.
- 4/27. The two left put into a big cage with 6 guinea-pigs which had been injected with tubercle bacilli 1 month before.
- 4/30. One of the young dead without lesions. Possibly trampled to death.
- 6/15. The injected guinea-pigs died. The young guinea-pig put into another cage along with guinea-pigs injected 20 days before.
- 8/28. The young one left was killed. No lesions.

This last result is exceedingly interesting, if it is confirmed by subsequent experiments.

- 22.—1/20/1915. A mother guinea-pig injected subcutaneously with 1 mg. of tubercle bacilli. Her two young, 2 days old, put with her.
- 2/9. Another fresh full-grown guinea-pig put into the same cage (small cage normally containing 2 guinea-pigs).
- 2/19. One of the young dead. No lesions.
- 2/24. Mother has ganglion and tumor at the point of inoculation.
- 3/26. The other young one dead. No lesions.
- 4/19. The third contact found dead. No lesions, but very emaciated. Mother used for other experiments.

The results of these experiments summed up indicate that the young had not suffered from tuberculosis at all, that they probably had attained immunity, tho I realize that I have not made enough experiments to warrant a conclusion.

#### CHANNEL OF INFECTION

A point which deserves investigation in connection with these experiments is how contact guinea-pigs become infected. Three hypotheses are possible: infection occurs (1) through the mouth, (2) through the nose, or (3) through both.

The following observations may enable us to discriminate between the three hypotheses: (1) Guinea-pigs never eat the hay which they have trampled on even when they are very hungry. (2) They never eat their own feces. (3) They never clean themselves as many other animals do. (4) When they are lively, they dig up the hay with closed mouth. (5) They never cough. (6) But when they are idle, they very often put themselves mouth against mouth.

The last observation suggested that the expired air contained the infective virus, and this is very probably the way in which they infect each other. It would mean that the channel of infection was chiefly the nose. The following experiments were designed to demonstrate this point:

- 23.—1/28/1915. One five-hundredths milligram of a 3-months-old culture put into the nose of each of 2 guinea-pigs.
- 3/ 1. One dead. Lesions in the spleen, which is of normal size, in the liver and lungs. Mesenteric ganglia.
- 3/ 8. The other killed. No macroscopic lesions.
- 24.—3/12/1915. Six guinea-pigs were instilled by way of the nose with 0.05 mg. of tubercle bacilli in one dose each. Divided into 3 lots of 2 each, A, B, and C.
- 3/16. Lot A left as control. Lots B and C were instilled again with the same quantity.
- 3/20. One of Lot B dead with pulmonary lesions only.
- 3/30. The two of Lot C instilled again with the same quantity.
- 4/28. One of Lot C dead. No macroscopic lesions.
- 8/ 6. Animals killed. A1—very small lesions in the lungs; A2—very many lesions in the lungs; B—lungs and spleen affected; and C—rare lesions in the lungs.

#### FEEDING EXPERIMENTS

- 25.— The same as before, except that the doses were doubled.
- 8/6/1915. Animals killed. No lesions.

#### CONCLUSIONS

Guinea-pigs can become infected through contact.

The infectivity follows a curve which is nil at the beginning of the infection, and goes on to the acme, and afterwards is no longer dangerous.

It seems that at the beginning of the process few tubercle bacilli are excreted, with which the new contact can easily deal, and acquire a kind of resistance which preserves the animal when the excretion is at its acme; but as soon as the infection goes on and the microbes swarm in the body, then massive doses are excreted. By this time the

new contact is powerless to cope at once with this large quantity, and therefore the pathogenic process establishes itself, and this is made worse by the daily absorption of great quantities of virus.

In these experiments, this condition arose between the 9th and the 33rd days after the infection.

After this period a puzzling condition was observed. The guinea-pigs no longer contracted the disease. It was assumed that at about this time the process of the encapsulation of the lesions began. Up to this time the virus had been wandering in the organism; after that period the organism had the power to form around the virus a wall which only let through the toxin, and perhaps a few microbes, or none, were excreted. Then the contacts could no longer be infected.

We have tried to give an experimental basis for the fact that the channel of infection in tuberculosis in guinea-pigs is chiefly the nose. In fact, extremely small doses which, given through the mouth, have no effect on the animals, given through the nose, almost certainly produce pulmonary tuberculosis.

We do not disregard the possible hypothesis that both channels of infection may help each other to bring the process more quickly to an end. It is known that small doses of tubercle bacilli repeatedly given through the mouth are more effective than a big dose given at one time. On the other hand, in our experiments small doses per os did not produce any trace of lesions, whereas the half of these given through the nose made guinea-pigs definitely tuberculous (Nos. 23, 24, and 25). Moreover, in these experiments the guinea-pigs, whether infected through the nose or by contact, presented pulmonary lesions. If we apply these results to our knowledge acquired in epidemiology in human beings, we must acknowledge that the second phase in our experiments is amply demonstrated. If we send tuberculous people to a country where there is no tuberculosis, the people of the country in question are affected in the same way as our guinea-pigs when they were put in contact with guinea-pigs already infected from the 9th to the 33rd day, and subsequently they die from an acute form of the disease and in a very short time.

As regards the first phase we have no observations to be compared with conditions obtaining among civilized people. This is an experimental condition which it would be difficult to obtain in observations of human beings. However, most probably we have similar conditions in people who excrete few tubercle bacilli. They infect their

neighborhood, but because of the small quantity excreted the contact organism can deal with it, and probably there arises that condition of immunity of which we hear frequently in human beings.

As regards the third phase, that guinea-pigs do not get infected in the last period, it is again difficult to find the corresponding phase in human beings, but we believe that there is probably a similar condition shown by cachectic tuberculous patients. At any rate, this is a problem which deserves inquiry, as also does that of the immunity arising in contacts.

# IMMUNE REACTIONS IN SCARLET FEVER, II\* ANTIGENIC PROPERTIES OF BACTERIA FOUND IN SCARLATINA

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In a previous report<sup>1</sup> the results of a number of complement-fixation tests in scarlet fever were given. In these tests the blood serum, spleen, and lymph glands, used as antigen, failed to indicate the presence of a specific virus. With mucus from scarlatinal angina as antigen, a weakly positive reaction was obtained in one instance. On the supposition that the antigenic property of the mucus was due to micro-organisms capable of growths, cultures were made of the mucus and tested as to antigenic properties by means of complement-fixation and intracutaneous reactions.

## COMPLEMENT-FIXATION

*Antigens 1, 2, and 3.*—On account of the evidence that scarlatina is at times transmitted by means of milk, cultures were made as follows:

A flask of sterile whey titrated to 0.5% acidity and a flask of sterile milk were inoculated with mucus from the throat of a scarlatinal patient who had been ill 2 days. After 4 days' incubation at 36 C. the milk had become coagulated. The whey was removed from it by passage through a Berkefeld filter, and the filtered whey added to the other whey, which contained a heavy growth of bacteria. The mixture was then heated at 60 C. for 1 hour.

This antigen was then employed in complement-fixation tests on the sera from 20 cases of scarlatina. A sheep-erythrocyte antiserum-rabbit-serum system was used with guinea-pig serum as complement; 2 units of amboceptor were used. By preliminary tests it was found that diluted antigen (1:1) in quantities of 0.05 c.c., 0.1 c.c., 0.15 c.c., and 0.2 c.c., gave the most satisfactory results; 0.2 c.c. of antigen usually gave slight inhibition. The scarlatinal serum was used at first in quantities of 0.025 c.c., 0.05 c.c., and 0.1 c.c., both active and inactivated. In later tests, however, 0.05 c.c. of the active serum only was used. By testing the hemolytic power of this serum corrections could be made so that the total complement and amboceptor were fairly constant in each test.

A control antigen (2) was made from mucus from the throat of a case of Ludwig's angina, the same method being employed as for the scarlatinal antigen. The scarlatinal sera were controlled by sera from cases giving no

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histories of scarlatina. These controls were uniformly negative. A third antigen was made by the method employed in the case of Antigen 1, mucus from the throat of another scarlatinal patient being used.

The results of these tests are shown in Table 1. Reactions are designated as + when slight inhibition was obtained in tubes containing one-fourth the quantity of antigen which alone showed inhibition. A well-marked inhibition is designated as ++, and complete inhibition as +++.

TABLE 1

RESULTS OF COMPLEMENT-FIXATION TESTS WITH ANTIGENS 1, 2, AND 3

Case and Description			Result of Test		
Number	Day of Disease	Temperature	Antigen 1 Scarlatinal Throat	Antigen 3 Scarlatinal Throat	Antigen 2 Ludwig's Angina
38	2	101	++	+	—
50	2	Normal	±	++	—
45	3	104.5	—	..	—
36	3	Normal	—	..	—
30	4	100.5	+++	..	++
33	4	Normal	+++	—	—
39	4	Normal	++	+	—
40	4	Normal	++	..	..
42	4	Normal	—	..	..
31	5	Normal	+++	..	..
41	7	Normal	+	..	..
35	11	Normal	++	..	..
34	12	Normal	+++	..	..
47	15	Normal	—	..	..
32	18	Normal	+++	..	..
47	22	Normal	—	..	..
44	23	Normal	—	..	—
37	24	Normal	++	..	—
36	27	Normal	++	..	..
49	31	Normal	±	..	++
43	32	Normal	—	..	+++
48	34	Normal	±	..	++

It will be seen from this table that with Antigen 1 fixation occurred in 12 of the 22 cases (54.6%). No fixation was obtained in 7 cases (31.8%), and in 3 cases the result was questionable (13.6%). With Antigen 3 a higher percentage of positive results was obtained, but the number of cases tested was small. Wassermann tests were negative in all the sera tried. With the antigen from Ludwig's angina, positive reactions occurred in one-third of the cases tried and negative reactions in two-thirds. The cultures from scarlatinal throats, then, showed the presence of an antigen which reacted with scarlatinal serum to fix complement in more than one-half the cases tested, whereas control antigen made from Ludwig's angina gave positive reactions in over one-third of the cases tested.

*Antigens 4, 5, and 6.*—With the object of finding the incubation period of the antigen present in the cultures of scarlatinal mucus the following experiment was made.

A flask of ordinary broth was inoculated with mucus from a scarlatinal throat. A part of this inoculated broth was at once heated to 60 C. for 1 hour and stored at 5 C. The remainder was incubated at 36 C. for 24 hours and again a portion removed and heated to 60 C. for 1 hour and stored at 5 C. The original flask was re-incubated for another 24 hours and then heated to 60 C. for 1 hour. In this way 3 antigens were obtained; one not incubated, one incubated 24 hours, and a third incubated 48 hours.

The three antigens were then examined by means of complement-fixation to see whether there was any increase of antigenic strength with incubation. These complement-fixation tests were carried out in a way similar to those described for Antigens 1, 2, and 3, 0.05 c.c. of complement and 2 units of amboceptor being used. The inactivated (at 56 C. for one-half hour) serum of a convalescent scarlatinal patient was used in the constant quantity of 0.1 c.c. in all the tests.

The result is shown in Table 2. The slight fixation obtained in the original broth-mucus mixture was increased in 24 hours to a strong fixation. In 48 hours the antigen alone began to inhibit more, while the fixation with scarlatinal serum was not further increased. The best antigen, therefore, was that of the 24-hour incubation.

TABLE 2  
ANTIGENIC STRENGTH OF BROTH CULTURES OF ANGINAL MUCUS AT THREE STAGES OF INCUBATION

Stage of Incubation		Quantities of Antigen Used					
		0.05 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.5 c.c.	1 c.c.
Before incubation, Antigen 4	{Antigen alone.....	—	—	—	—	—	+
	{With scarlatinal serum	—	—	—	+	..	..
After 24 hours, Antigen 5....	{Antigen alone.....	—	—	—	—	—	+
	{With scarlatinal serum	—	—	++	+++	..	..
After 48 hours, Antigen 6....	{Antigen alone.....	—	—	—	±	±	+
	{With scarlatinal serum	—	—	++	+++	..	..

*Antigen 7.*—Examinations of Antigens 1 to 6 showed that the predominant growth in 24 hours was that of streptococci. In order to compare the antigenic property of streptococci with that of the mixed cultures of the preceding experiment, a hemolytic streptococcus was isolated from scarlatinal angina and a broth antigen prepared as in the case of Antigens 4, 5, and 6.

With the same technic as before tests were carried out in 9 cases of scarlatina. Of these 9 cases, the sera of 8 (88%) showed fixation of complement, which in 4 instances was complete. These results would indicate that no antigenic property need be assumed in any of the antigens prepared from scarlatinal throats other than that of the streptococci present.

TABLE 3

COMPARISON OF THE ANTIGENIC STRENGTHS OF CULTURES OF THE VARIOUS ORGANISMS IN ANGINAL MUCUS

Serum	Antigen										
	7	8	9	10	11	12	13	14	15	16	17
18	..	..	..	..	..	..	..	..	..	..	..
20	+	..	+	+	..	..	..	..	+	..	..
21	..	..	..	..	+++	+++	+	..	..	..	..
22	..	++	..	..	..	..	..	++	..	++	++
23	++	..	+	+	..	..	..	..	..	..	..
24	+++	..	—	+	..	..	..	..	..	..	..
25	—	..	—	—	..	..	..	..	..	..	..
26	++	..	—	—	..	..	..	..	..	..	..
27	+	..	—	—	..	..	..	..	..	..	..
28	+++	..	—	+	..	..	..	..	..	..	..
29	+++	..	—	+	..	..	..	..	..	..	..
30	+++	..	—	++	..	..	..	..	..	..	..
31	..	..	..	..	..	..	..	..	..	..	..
32	..	..	..	..	..	..	..	..	..	..	..
33	..	..	..	..	..	..	..	..	..	..	..
34	..	..	..	..	..	..	..	..	..	..	..
35	..	..	..	..	..	..	..	..	..	..	..
36	..	..	..	..	..	..	..	..	..	..	..
37	..	..	..	..	..	..	..	..	..	..	..
38	..	..	..	..	..	..	..	..	..	..	..
39	..	..	..	..	..	..	..	..	..	..	..
40	..	..	..	..	..	..	..	..	..	..	..
41	..	..	..	..	..	..	..	..	..	..	..
42	..	..	..	..	..	..	..	..	..	..	..

\* Control sera from convalescent diphtheria cases +++ and +.

† Control sera from convalescent diphtheria cases +++ and ±.

*Other Antigens.*—In order to compare the antigenic property of streptococci with that of other organisms from scarlatinal anginas, a number of organisms were isolated in pure culture and antigens prepared from them. When possible these antigens were prepared in the same manner as the preceding ones, but in some cases other cultural methods were necessary. A brief description of these organisms follows:

8. Streptococcus similar to that of Antigen 7.

9. Bacillus: Slender, tapered, with tendency to slight curve; gram-negative; aerobic. Good growth on ordinary media, mannite, milk, lactose, and dextrose; unchanged at end of 48 hours. Slight green discoloration produced in blood agar. Growth formed a grayish-yellow, very moist film.

10. Micrococcus tetragenus.

11. Bacillus: Large; spore-forming; gram-positive; aerobic. Good growth on ordinary media; opaque cream-colored moist growth on blood agar.

12. *Bacillus*: Small; gram-positive; aerobic. Opaque white growth on blood agar; color of blood changed to green.

13. *Bacillus*: Small; gram-positive; ends tapered; tendency to slight curve, especially where it occurs in pairs; aerobic. White opaque moist film on blood agar; slightly hemolytic.

14. *Diplobacillus*: Gram-positive; aerobic; hemolytic. Convex brownish opaque colonies on blood agar.

TABLE 3—Continued

COMPARISON OF THE ANTIGENIC STRENGTHS OF CULTURES OF THE VARIOUS ORGANISMS IN ANGINAL MUCUS

Antigen											
18	19	20	21	22	23*	24	25	26	27	28†	29
..	..	..	..	..	..	..	—	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..
—	+	—	..	..	..	..	..	—	..	..	..
—	—	—	+	—	..	..	..	—	..	..	..
—	+	—	—	+	..	..	..	—	..	..	..
—	—	—	—	..	..	..	..	—	..	..	..
—	—	—	—	—	..	..	..	..	..	..	..
—	—	—	..	—	..	..	..	..	..	..	..
—	—	—	..	—	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	—	..	..
..	..	..	..	..	..	..	..	..	+++	..	..
..	..	..	..	..	..	..	..	..	+++	..	..
..	..	..	..	..	..	..	..	..	±	..	..
..	..	..	..	..	..	—	..	..	..	—	..
..	..	..	..	..	..	—	..	..	..	—	..
..	..	..	..	..	—	..	..	..	..	..	..
..	..	..	..	..	—	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..

15. *Bacillus*: Small; gram-positive; occurring in chains; anaerobic; hemophilic. Blood unchanged; grayish translucent film on blood agar.

16. *Bacillus*: Very small; gram-negative; anaerobic. No growth on ordinary media; no change in blood; pinpoint transparent colonies on human-blood agar.

17. *Bacillus*: Small, with great variation in length; tendency to occur in chains; some of longer forms showed vacuoles; gram-negative; anaerobic. No growth on ordinary media; delicate transparent film on human-blood agar; slight but constant hemolysis. Specimens stained with methylene blue somewhat resembled diphtheria bacillus.

18. *Bacillus mucosus*: Short; encapsulated; gram-negative; aerobic; mucoid growth; hemolytic. Dextrose, mannite, and lactose acid; milk acid and coagulated.

19. *Bacillus*: Formed chains; gram-positive; aerobic. Small convex grayish opaque colonies or yellowish opaque raised film; good growth on ordinary media; dextrose, lactose, and mannite acid; milk acid but not coagulated; blood hemolyzed.

20. *Bacillus*: Spore-forming; gram-positive; aerobic. Formed dry finely wrinkled film on surface of plain agar; hemolytic.

21. *Streptobacillus*: Gram-positive; anaerobic; hemolytic. Lactose, mannite, and dextrose-litmus agar bleached to canary-yellow color; litmus milk bleached and not coagulated; grayish transparent film on surface of blood agar.

22. *Bacillus mucosus* similar to No. 18.

23. *Coccus*: Oval; occurred in pairs; did not form chains; gram-positive; aerobic. Grew on ordinary media; no change in dextrose, lactose, mannite-litmus agar, litmus milk, or blood agar.

24. *Streptothrix*: Branched thread-like organism; gram-positive; anaerobic. No growth on ordinary media; growth on human-blood agar slow, forming transparent film at end of 48 hours, which at the end of 4 days changed to brownish opaque layer; single colonies became umbilicated.

25. *Bacillus*: Very small; gram-negative; anaerobic. No growth on ordinary media; blood unchanged.

26. *Bacillus*: Thread-like; gram-negative; anaerobic. No growth on ordinary media; growth on human-blood agar scant, forming transparent film without change in the blood.

27. *Bacillus*: Varying from short coccoid form to thread-like structure; encapsulated; aerobic; characterized by irregular unstained areas and mucoid nature of growth; gram-negative.

28. *Bacillus*: Very small; gram  $\pm$  at end of 24 hours; aerobic. Numerous minute grayish translucent colonies on blood agar; no effect on blood.

29. *Diphtheroid*: Gram-positive; aerobic; pleomorphic. Yellowish-gray raised growth; good growth on ordinary media.

It is evident from Table 3 that the sera tested may be divided into 2 types: (1) sera which tend to fix complement with a number of antigens, such as Sera 20 and 21; and (2) sera which show little tendency to fix complement with any antigen, such as Sera 25 and 27.

Livierato,<sup>2</sup> in a study of the antibodies developed during the acute exanthemata, divided them into specific and associated antibodies. For instances, in 10 cases of erysipelas, 2 cases showed antibodies for streptococcus alone, 2 for staphylococcus, 1 for pneumococcus, 1 for streptococcus and staphylococcus, 2 for streptococcus and gonococcus, 1 for streptococcus and the typhoid bacillus, 1 for streptococcus, staphylococcus, and pneumococcus. In measles, peliosis rheumatica, variola, and varicella, similar associated antibodies were found. Livierato failed to find associated antibodies in scarlet fever. This may have been due to the organisms used as antigens. The fixation of complement with a number of antigens may indicate a development of nonspecific immune bodies or the development of specific antibodies to a number of organisms. The only organism tested which appears to produce immune bodies with any degree of constancy in scarlatina is the streptococcus. This is in accord with the results of Tunnicliff,<sup>3</sup>

<sup>2</sup> Gazz. d. osp. Milano, 1907, 28, p. 835.

<sup>3</sup> Jour. Infect. Dis., 1907, 4, p. 304.

Schleissner,<sup>4</sup> and others. Kolmer,<sup>5</sup> however, found fixation of complement with streptococcus antigen in only 11.2% of sera from scarlet-fever convalescents.

#### CUTANEOUS TESTS

Cutaneous tests on convalescent scarlet-fever patients were carried out with antigens made as for the complement-fixation tests. As controls, diphtheria patients were used who gave no history of scarlet fever. The technic used was similar to that of the intracutaneous tuberculin and Schick tests. Preliminary experiments with the von Pirquet scarification technic were unsatisfactory. The antigen was a heat-sterilized culture of mucus from scarlatinal angina.

In 12 scarlet-fever patients there developed at the site of injection areas of reddening and induration which reached a maximum on the day following the injection and then gradually disappeared. The controls showed much less marked reactions.

In order further to investigate this reaction the following study was made. Three antigens were prepared: (1) a milk and whey culture of mucus from scarlatinal angina; (2) a pure culture of a hemolytic streptococcus from a fatal scarlatinal angina; (3) a milk and whey culture from an ordinary follicular tonsillitis due apparently to streptococcal infection. The three antigens were injected intracutaneously on the outer aspect of the upper arm about 4 cm. apart. In this way the reactions following the injection of the three antigens were compared with each other and with control reactions obtained in the same way in diphtheria convalescents. One-tenth cubic centimeter of fluid containing, according to turbidity, about the same quantity of bacteria was used for each injection. Accurate measurements of the areas of reaction which occurred are shown in Table 4. It will be noted that in some instances one diphtheria control served for more than one of the 10 scarlet-fever patients.

The reactions in the scarlatinal convalescents were as follows:

*Scarlatinal-Throat Antigen.*—An area of reddening of the skin developed in a few hours and reached its height in about 24 hours, when it averaged about 2 cm. in diameter. At the end of the 2nd day the redness began to disappear and was gone by the 3rd or 4th day. The reddening was accompanied by an induration, which developed a little more slowly, but also reached a maximum in about 24 hours,

<sup>4</sup> Wien. klin. Wchnschr., 1909, 22, p. 553.

<sup>5</sup> Arch. Int. Med., 1912, 9, p. 220.

TABLE 4  
CUTANEOUS REACTIONS IN SCARLATINA

Case	Disease and Day of Disease	Antigen	Reaction (cm.) 1st Observa- tion		Reaction (cm.) 2nd Observa- tion		Reaction (cm.) 3rd Observa- tion	
			Redden- ing	Indura- tion	Redden- ing	Indura- tion	Redden- ing	Indura- tion
F. O. 12 years	Diphtheria 1 month	Scarlet fever Streptococci Tonsillitis	5 hr.  2	5 hr.	28 hr. 0.8×0.5 1.7×1.8 1.5	28 hr. 0.5 1.5 0.7	50 hr. 0.5×0.6 0.7×0.6 0.2	50 hr. 0.2 0.8
M. R. 12 years	Scarlet fever convales- cence 5 weeks	Scarlet fever Streptococci Tonsillitis	5 hr. 1 3	5 hr. 1 3.5 edema	28 hr. 1.2×1 2.5 7×8	28 hr. 0.5×1.0 1 7×8 edema	50-72 hr. 0.7×6 1×1.4 0.5	50-72 hr. 0.7×0.5 0.8×1 1
E. W. 3 years	Scarlet fever and diph- theria 3 weeks	Scarlet fever Streptococci Tonsillitis	5 hr. 0.5 0.2 1	5 hr. 1 0.5 1	28 hr. 0.3 0.3 1.7	28 hr. 0.5 0.3 1.0	50-74 hr.	50-74 hr. 0.5 0.5
M. P. 9 years	Scarlet fever 3 weeks	Scarlet fever Streptococci Tonsillitis	3 hr. 1	3 hr. 1 edema	5 hr. 1	5 hr. 1 edema	26 hr. 6 1 3	26 hr. 1 0.5
M. K. 8 years	Diphtheria convales- cence	Scarlet fever Streptococci Tonsillitis	3 hr. 0.5 0.3 0.7	3 hr. 0.5 edema 0.7	5 hr. 0.5 0.3 0.7	5 hr. 0.5 edema 0.7	26 hr. 2.5 2.5 5	26 hr.  0.2
B. N. 7 years	Diphtheria convales- cence 26 days	Scarlet fever Streptococci Tonsillitis	4 hr. 0.5 Injection failure 1.2×3	4 hr. 1.2×3 edema	24 hr. 1.0 2.0×3.5	24 hr. 0.7 2.0×3.5		
M. A. 21 years	Scarlet fever convales- cence 20 days	Scarlet fever Streptococci Tonsillitis	4 hr. 0.5 faint 0.5 faint 3	4 hr.	24 hr. 4.0×4.5 3×4 5×6	24 hr. 0.5 0.5		
J. H. 6 years	Scarlet fever convales- cence 7 days	Scarlet fever Streptococci Tonsillitis	4 hr. 0.5×0.7 0.3 3	4 hr.	24 hr. 0.5 0.5 2×2.5	24 hr. 0.5 0.5 0.5		
K. H.	Scarlet fever 27 days	Scarlet fever Streptococci Tonsillitis	5 hr.  2×2.5 1.5×0.5 2×2.5	5 hr.  2.5 0.2 1.5	28 hr.  3×3.5 1.5 3×4	28 hr.  1.5 1 1.5	72 and 96 hr. 1.5 1	72 and 96 hr. 1.5 0.5
H	Scarlet fever 28 days	Scarlet fever Streptococci Tonsillitis	5 hr.	5 hr.  2.5 2.2 1.0	28 hr.  1.5×2 1.5×2	28 hr.  1.0 0.2	72 and 96 hr. Slight	72 and 96 hr. 1
A	Diphtheria convales- cence	Scarlet fever Streptococci Tonsillitis	5 hr.	5 hr.	28 hr.  0.5 0.5	28 hr.  0.5 0.5	72 and 96 hr.	72 and 96 hr.
Mr. M. 25 years	Scarlet fever 12 days	Scarlet fever Streptococci Tonsillitis	3 and 5 hr.	3 and 5 hr. 0.5 0.2	50 hr.	50 hr.	72 hr.	72 hr.
E. M.	Scarlet fever streptococcus mastoiditis 3 mo.	Scarlet fever Streptococci Tonsillitis	3 and 5 hr.	3 and 5 hr. 0.5	50 and 74 hr. 0.5	50 and 74 hr. 0.5	96 hr. hr.	96 hr. hr.
A. M. 2 years	Diphtheria- carrier 3 mo.	Scarlet fever Streptococci Tonsillitis	3 and 5 hr.	3 and 5 hr.	50 hr.	50 hr.  0.2 0.2 0.2		
R. S. 12 years	Scarlet fever 30 days	Scarlet fever Streptococci Tonsillitis	4 hr. 1.0 0.5	4 hr.	24 hr. 2 3 Slight	24 hr. 0.5 2.0 0.3		

when it averaged about 1 cm. in diameter. After 48 hours the induration began to disappear and by the end of a week was usually gone.

*Streptococcus Antigen*.—The reaction was similar to that with the scarlatinal-mucus antigen except that the average diameter of the area of reddening was about 1.5 cm. and the diameter of the induration about 0.5 cm.

*Tonsillitis Antigen*.—With the tonsillitis antigen more variation occurred than with either of the other antigens. The area of redness was usually larger, being about 3 cm., and the induration averaged about 0.5 cm.

The reactions in the convalescent diphtheria patients developed more slowly than those in the scarlatinal patients. The reddening was not pronounced until the day following. The period of maximal development, however, was about the same as in the scarlatinal cases. The induration did not, as a rule, last so long. The average measurements at the time of maximal development were as follows: (1) scarlatinal throat antigen, reddening 4 cm., induration 0.5 cm.; (2) streptococcus antigen, reddening 0.75 cm., induration 0.5 cm.; (3) tonsillitis antigen, reddening 0.7 cm., induration 0.5 cm.

The interpretation of the cutaneous reactions offers some difficulty, but the reaction of the skin in convalescent scarlatinal patients to all three antigens was much more marked than the reaction of the diphtheria skin. In this connection an observation made by Heim<sup>6</sup> is of interest. He made von Pirquet tests for tuberculosis with 3 dilutions in a child who gave positive reactions to all three dilutions. He then, in the course of the following week, gave 3 subcutaneous injections of small quantities of tuberculin, none of which produced local reactions. A month after the tuberculin tests were made, the child developed scarlet fever and at the same time severe reactions occurred at the sites of all 6 tuberculin inoculations. Aside from this general hypersusceptibility to irritation of the convalescent scarlatinal skin, the significance of the skin reactions is not clear. There was, as a rule, a greater difference between the indurations following the injection of the scarlatinal-mucus antigen in scarlatinal patients and control than between the indurations caused by either of the other two antigens in scarlatinal patients and controls. On the other hand the scarlatinal skin gave more marked reddening after the injection of tonsillitis antigen than after the injection of scarlatinal antigen and streptococcus. A specificity comparable to the cutaneous tuberculin test could not be demonstrated.

<sup>6</sup> Wien. med. Wchnschr., 1908, 58, p. 1831.

# THE PRESENCE OF SOIL AND FECAL STRAINS OF ORGANISMS OF THE COLON-AEROGENES GROUP IN THE WATERS OF KANSAS \*

MYRTLE GREENFIELD

*From the Water and Sewage Laboratory of the Kansas State Board of Health, Lawrence*

It was demonstrated by Rogers, Clark, and Evans<sup>1</sup> that organisms in the colon-aerogenes group occurring on grains may be differentiated from those of fecal origin by the gas ratio. Clark and Lubs<sup>2</sup> showed that there is a complete correlation between the gas ratio and the hydrogen-ion concentration, the fecal strains in their media being characterized by a high hydrogen-ion concentration, the cultures from grains by a low hydrogen-ion concentration. The difference in H<sup>+</sup> concentration is easily recognized with methyl red as an indicator.

If organisms which apparently are of the colon-aerogenes group occur on grains and in soil, then their presence in water cannot be taken as evidence of fecal contamination unless it is proved that the fecal strains are more prevalent than the soil strains. It is the pur-

## ERRATUM

Paper by Greenfield on the presence of soil and fecal strains of organisms of the colon-aerogenes group in the waters of Kansas, Vol. 19, No. 4, p. 647, the last sentence on the page should read: "All cultures that did not ferment dextrose and lactose, produce acid, and coagulate milk, and which liquefied gelatin, were discarded."

fermentation tubes, to the dipotassium-acid-phosphate medium of Clark and Lubs,<sup>2</sup> and to litmus milk and gelatin tubes. The fermentation tubes and milk tubes were incubated 2 days at 37 C., and the dipotassium-acid-phosphate medium was incubated 5 days at 30 C. One half of the latter was tested with methyl red and the other half with 10% KOH to obtain the Voges-Proskauer reaction. All cultures that did not ferment dextrose and lactose, produce acid, and coagulate milk, and which failed to liquefy gelatin, were discarded.

\* Received for publication June 19, 1916.

<sup>1</sup> Jour. Infect. Dis., 1915, 17, p. 137.

<sup>2</sup> Ibid., p. 160.

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If organisms which apparently are of the colon-aerogenes group occur on grains and in soil, then their presence in water cannot be taken as evidence of fecal contamination unless it is proved that the fecal strains are more prevalent than the soil strains. It is the purpose of this paper to record the prevalence of the two strains of *B. coli* in ground waters and surface waters in the state of Kansas.

## SOURCES OF CULTURES AND METHOD OF ISOLATION

Of the cultures studied, 247 were isolated from surface waters, 158 from ground waters, 19 from natural ice, and 8 from manufactured ice.

Both broth and bile fermentation tubes, made according to the Standard Methods, were used in the presumptive test for organisms of the colon-aerogenes group. From April 4 to May 4, 1916, a fermentation tube of every positive sample was saved and the sample streaked on Endo agar. Colonies having the appearance of *B. coli* were picked and streaked on agar slants. From these, transfers were made to dextrose, lactose, saccharose, and dulcitol fermentation tubes, to the dipotassium-acid-phosphate medium of Clark and Lubs,<sup>2</sup> and to litmus milk and gelatin tubes. The fermentation tubes and milk tubes were incubated 2 days at 37 C., and the dipotassium-acid-phosphate medium was incubated 5 days at 30 C. One half of the latter was tested with methyl red and the other half with 10% KOH to obtain the Voges-Proskauer reaction. All cultures that did not ferment dextrose and lactose, produce acid, and coagulate milk, and which failed to liquefy gelatin, were discarded.

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<sup>1</sup> Jour. Infect. Dis., 1915, 17, p. 137.

<sup>2</sup> Ibid., p. 160.

CHARACTERISTICS OF THE CULTURES

The distribution of the cultures among MacConkey's<sup>3</sup> 4 principal groups and their relation to methyl red are shown in Table 1. This table shows 70% of the total number isolated to be acid to methyl red, that is, of fecal origin.

TABLE 1  
DISTRIBUTION OF ORGANISMS ISOLATED FROM SURFACE WATERS AND GROUND WATERS

Organism	Number	Acid to Methyl Red	Percentage	Alkaline to Methyl Red	Percentage
B. communior.....	186	142	76	44	24
B. communis.....	50	44	89	6	11
B. aerogenes.....	112	54	49	58	51
B. acidi-lactici.....	57	42	74	15	26
Total.....	405	282	70	123	30

Table 2 shows the distribution of cultures isolated from raw water, including river, creeks, and impounding reservoirs.

TABLE 2  
DISTRIBUTION OF ORGANISMS ISOLATED FROM RAW WATER

Organism	Number	Acid to Methyl Red	Percentage	Alkaline to Methyl Red	Percentage
B. communior.....	56	47	84	9	16
B. communis.....	15	13	87	2	13
B. aerogenes.....	29	18	62	11	38
B. acidi-lactici.....	16	10	63	6	37
Total.....	116	88	76	28	24

Table 3 shows the distribution of cultures isolated from treated waters. Most of these were waters filtered through mechanical filters; the others were waters settled with alum, or lime and alum only, or waters treated with hypochlorid.

TABLE 3  
DISTRIBUTION OF ORGANISMS ISOLATED FROM TREATED WATER

Organism	Number	Acid to Methyl Red	Percentage	Alkaline to Methyl Red	Percentage
B. communior.....	62	50	81	12	19
B. communis.....	19	16	84	3	16
B. aerogenes.....	31	14	45	17	55
B. acidi-lactici.....	19	12	63	7	37
Total.....	131	92	70	39	30

<sup>3</sup> Elements of Water Bacteriology, 1915, p. 149.

A slightly higher percentage of the cultures from raw water were of fecal origin than of the cultures from treated waters. Samples of raw and treated waters were taken at the same time, but the raw waters were practically always positive in the presumptive test while many of the treated waters were negative. During the month that these cultures were isolated there was a high rainfall over almost the whole of Kansas. It would seem that there would be a higher percentage of *B. coli* of soil origin during high-water periods than during low-water periods.

Daily tests in the field are now being made at Cherryvale, Independence, Coffeyville, Chanute, Humboldt, and Washington, all surface supplies, to find whether one strain of *B. coli* is more resistant than another in the treated waters, and whether there is a recurrence of the organism in MacConkey's 4 principal groups.

Experiments, started more than 2 years ago, are being conducted in this laboratory to determine the longevity of organisms of the colon-aerogenes group in soil. Organisms of both soil and fecal origin are being used under both natural and artificial conditions.

TABLE 4  
DISTRIBUTION OF ORGANISMS ISOLATED FROM ICE

Organism	Number	Acid to Methyl Red	Percentage	Alkaline to Methyl Red	Percentage
ORGANISMS FROM NATURAL ICE					
<i>B. communior</i> .....	10	3	100	0	70
<i>B. communis</i> .....	1	1	100	0	0
<i>B. aerogenes</i> .....	7	1	14	6	86
<i>B. acidi-lactici</i> .....	1	1	100	0	0
Total.....	19	6	32	13	68
ORGANISMS FROM MANUFACTURED ICE					
<i>B. communior</i> .....	2	1	50	1	50
<i>B. communis</i> .....	4	4	100	0	0
<i>B. aerogenes</i> .....	1	0	0	1	100
<i>B. acidi-lactici</i> .....	1	1	100	0	0
Total.....	8	6	75	2	25

Of the cultures from raw water 76% were of fecal origin, while of the organisms from natural ice only 32% were of fecal origin. Therefore if so small a number as 19 can be considered, it would seem that organisms of fecal origin are much shorter lived in ice than those of soil origin.

TABLE 5  
DISTRIBUTION OF ORGANISMS ISOLATED FROM GROUND WATERS

Organism	Number	Acid to Methyl Red	Percentage	Alkaline to Methyl Red	Percentage
<i>B. communior</i> .....	68	45	66	23	34
<i>B. communis</i> .....	16	15	94	1	6
<i>B. aerogenes</i> .....	52	22	43	30	57
<i>B. acidilactici</i> .....	22	20	91	2	9
Total.....	158	102	65	56	35

Table 5 gives the distribution of 158 cultures isolated from ground waters. Sixty-five percent of the cultures were of fecal origin. Of the total, 123 were isolated from wells or springs in areas where the rock formation is limestone. Of these, 77% were of fecal origin. Thirty-four of the cultures were from wells or springs in areas where the rock formation is sandstone, shale, or glacial drift. Of these only 50% were of fecal origin.

#### CORRELATION OF THE METHYL RED AND VOGES-PROSKAUER REACTIONS

The work of Max Levine<sup>4</sup> on 9 cultures from sewage and 4 cultures from the American Museum of Natural History indicates that there is a complete correlation between the positive Voges-Proskauer reaction and the alkaline methyl-red reaction. The work I have done on cultures isolated from the waters and ices of Kansas agrees with his work. Of the cultures 138 were alkaline to methyl red, that is, they were of soil or grain origin, and in every instance they gave the positive Voges-Proskauer reaction. None of the cultures that were acid to methyl red, that is, of fecal origin, gave a positive Voges-Proskauer reaction.

#### CONCLUSIONS

The work on 247 cultures isolated from raw and treated waters indicates that a slightly higher percentage of the cultures from raw water were of fecal origin, than of the cultures from treated waters.

The work on 19 cultures from natural ices, as compared with 116 cultures from raw waters, would seem to indicate that colon bacilli of soil origin are more resistant to zero temperature than those of fecal origin.

<sup>4</sup> Jour. Infect. Dis., 1916, 18, p. 358.

A higher percentage of cultures of the colon-aerogenes group from wells and springs in limestone formation were of fecal origin than of those from wells and springs in sandstone, shale, and glacial drift. Too much emphasis should not be given this, however, since the number of wells in sandstone, shale, and glacial drift was limited.

The work on 405 cultures from surface and ground waters shows that 70% of the organisms of the colon-aerogenes group were of fecal origin.

There is complete correlation between the positive Voges-Proskauer reaction and alkalinity to methyl red.

# THE MILKING MACHINE A SOURCE OF BACTERIAL CONTAMINATION OF MILK \*

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In some localities hand milkers are being rapidly replaced by more or less complicated machines that do the milking in what has been assumed to be the most sanitary way. Some of this machinery, however, is not very easily kept clean and may therefore become a source of contamination to rival the most careless hand milker.

In a recent bacteriologic study of the milk delivered to the retailers in LaSalle and Peru I found that those milks which had been drawn by a milking machine almost invariably gave higher bacterial counts than those which had been drawn by hand. This fact is clearly brought out by the following table of the average counts for 2 months :

TABLE 1

THE AVERAGE BACTERIAL COUNTS FOR MILKS DELIVERED TO THE RETAILERS IN LASALLE AND PERU DURING MAY AND JUNE, 1916

Dairy (Milk Drawn by Machinery)	Number of Bacteria per c.c.	Dairy (Milk Drawn by Hand)	Number of Bacteria per c.c.
B	2,733,000	E	1,795,000
F	2,823,000	En	52,500
G	1,435,000	Ma	113,000
J	1,195,000	Me	270,000
M	2,630,000	R	140,000
S	785,000	Sr	315,000

The table shows plainly that milk drawn with a milking machine may give a much higher bacterial count than milk of the same age which has been drawn by hand. It should be stated, however, that this difference is not generally so marked during cold weather as during warm weather.

These rather surprising results led me to visit every dairy in this vicinity where a milking machine is used, with a view to determining just where the trouble lay. In this investigation sterile wide-mouthed bottles in a small ice box were taken out to the farms and samples collected at different stages of the handling of the milk, for bacteriologic examination. As a rule 2 cows were milked at the same time

\* Received for publication, July 15, 1916.

with 1 milking machine. Before the milking machine was started, we drew a small quantity of milk from 2 cows into a sterile bottle and immediately placed it on ice. The cups of the machine were then put on those cows, and after they were milked dry, another sample was poured into one of our sterile bottles and placed on ice. The teat cups, tubes, and milking can of the milking machine were then rinsed with clean cold water and sterilized by drawing a pailful of boiling hot water through them. After the cups had cooled sufficiently so that they could be attached to the teats, a second pair of cows was milked with the machine and a sample poured into a sterile bottle and placed on ice. These samples were then immediately transported to the laboratory, where they were plated in 1% acid agar and the colonies counted after incubation at 36 C. for 48 hours. The results, which were very striking, are shown in Table 2.

TABLE 2

COMPARISON OF THE BACTERIAL COUNTS FROM MILK DRAWN BY HAND, BY UNSTERILIZED MILKING MACHINES, AND BY MACHINES THOROUGHLY SCALDED

The Manner in Which Milk Was Drawn	Dairy B	Dairy F	Dairy G	Dairy J	Dairy M	Dairy S
By hand into a sterile bottle.....	860	.....	556	1,200	11,600	12,800
With milking machine as generally used .....	2,450,000	190,000	432,000	320,000	660,000	266,000
With same milking machine after thorough scalding of the teat-cups, tubes, and can with boiling water .....	2,430	12,500	16,000	5,000	130,000	6,000

The figures show clearly that milk may be grossly contaminated by the use of an improperly cleaned and unsterilized milking machine. These farmers had been instructed by the agent who sold them the milking machine to rinse the cups, tubes, and cans with cold water after each milking and then to lay the tubes and cups into a tub of clean water until the next milking. This was done just as they had been instructed except that some of them placed the tubes and cups into a solution of "B-K" or a solution of chlorinated lime. The water or solution in these tubs was changed twice a week, and once each week the cups and tubes were cleaned with a brush and "scalded." The "scalding," however, was done with water that was only hot enough to "feel hot" to the finger.

It is a well known fact that milk utensils and rubber tubes through which milk has been drawn cannot be properly cleaned by merely rinsing with cold water. When we consider the fact that this milk was

drawn through rubber tubes which are about 3 feet long and which have several connecting joints, and that they were cleaned only once a week, we are not greatly surprised at the results. The filthiness of the connecting joints and of the interior of these tubes in hot weather can easily be imagined.

#### CONCLUSION

Milk may be badly contaminated by a milking machine if the teat cups and rubber tubes are not carefully cleaned and scalded before each milking.

Immersing the cups and tubes in a solution of "B-K" or chlorinated lime does not satisfactorily prevent bacterial growth in the tubes.

A mere inspection of the dairy without bacteriologic control of the milk may fail absolutely to locate an unsanitary process in the production of that milk. All our dairies are inspected by a veterinarian every 3 months and are given fairly high scores, but he has never discovered the fact that the milking machines are not properly cleaned and scalded.

# THE SPECIFICITY OF THE ABDERHALDEN REACTION WITH VEGETABLE PROTEINS\*

## THE BIOLOGIC REACTIONS OF THE VEGETABLE PROTEINS, VIII

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It is now generally assumed that when a protein is introduced parenterally into the animal organism, antibodies are elaborated within the latter which lead to the destruction of that protein. Vaughan<sup>1</sup> believes this reaction to be universal enough to be incorporated into a biologic law. The nature of these antibodies, the seat of their formation, and the mode of their action are as yet unanswered questions. They are thought, however, to be able to cause cleavage of the protein molecule introduced, and the products of this cleavage may cause certain phenomena, which can be detected by the characteristic behavior of the animal employed. Anaphylaxis is an example of such phenomena. As early as 1910 Abderhalden attempted to establish the nature of the proteolytic ferments concerned in these reactions—protective ferments he called them. After adding some of the immunized animal's serum to the specific antigen in vitro, he noted as the result of protein digestion the appearance of dialyzable products, which he detected by various methods. He stated, moreover, that these ferments were specific and represented one of the responses of the animal body to the protein introduced. From these observations Abderhalden developed his reaction for the diagnosis of pregnancy. Abderhalden assumed that the complex cells of the chorionic villi enter the maternal blood stream during pregnancy and are there rendered innocuous by specific enzymes especially generated for that purpose. The presence of these so-called specific or protective ferments, just as in animal experimentation, could be demonstrated in vitro by adding a definite amount of the patient's serum to some of the specific protein assumed to be acting as antigen in this case, placenta. Specificity was likewise considered as demonstrated by the detection, by the same methods, of dialyzable products resulting from the interaction of serum and sub-

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<sup>1</sup> Protein Split Products in Relation to Immunity and Disease, 1913, p. 25.

strate. The serum was thought to be unable to interact with any other substrate than placental protein. Apparently, then, these ferments develop whether the protein enters the blood stream parenterally from within, or from without, the animal body. In either case they were held by him to be specific and to be demonstrable by the same methods.

Abderhalden's application of his theory of protective ferments and his methods for detecting the presence of the latter have stimulated a vast amount of work, and consequently a very extensive literature has developed. Since there are at present several elaborate reviews of this literature, it is deemed unnecessary to add another here. It is probably sufficient to state that there is no concordance of opinion among investigators as to the mechanism or the specificity or nonspecificity of the reaction.

The bulk of experimental work has been done with animal materials obviously more or less complex as to nature and composition. The use of especially prepared pure isolated proteins instead, such, for example, as are obtained from vegetable sources, seemed highly desirable, since it would tend to eliminate many serious and noteworthy objections. If the animal body responds to the parenteral introduction of a mixture of proteins by the generation of ferments more or less specific, it should be able to do so all the more specifically on the introduction of a single pure and extremely foreign protein. With the intent, then, of removing some of the uncertainty now existing as to the specificity of the Abderhalden reaction, this work was undertaken using as antigen carefully purified preparations of vegetable proteins obtained from Dr. T. B. Osborne. These proteins, furthermore, offer the added advantage of having been extensively studied by other immunologic methods, by Osborne, Wells, Lake, and White.<sup>2</sup>

The methods employed in making the preparations of the vegetable proteins used in this investigation and the chief chemical characteristics of these substances have been described by Osborne.<sup>3</sup> The immunologic reactions produced by them have also been discussed by Wells and Osborne in earlier papers of this series. Crude extracts of plant seeds have been employed with success for similar purposes by earlier workers. Both the isolated vegetable proteins and the plant extracts have also been used to some extent in immunizing animals for the Abderhalden test. Abderhalden, injecting gliadin, edestin, and casein,

<sup>2</sup> Jour. Infect. Dis., 1911, 8, p. 66. This paper also contains an historical review of the use of plant extracts in immunologic reactions. Ibid., 1913, 12, p. 341; 13, p. 103; 1914, 14, pp. 364, 377; 1915, 17, p. 259; 1916, 19, p. 183.

<sup>3</sup> Ergebn. d. Physiol., 1910, 10, p. 47.

and peptone from gelatin, edestin, and casein, obtained ferments which he maintained are specific for those substances. Issatschenko obtained similar results, using as antigen extracts of hempseed, wheat flour, hazelnut, and oats. He found that the serum decomposed the proteins in the extracts used for injection, but not the others. However, the immune serum would not differentiate between extracts from two closely allied plants, such as two species of flax, when the one was employed as antigen. In this sense the serum was not specific. Denying entirely the specificity of the Abderhalden reaction, Herzfeld<sup>4</sup> found that the sera of all normal, pregnant, and neurotic persons investigated by him were capable of digesting zein, the alcohol-soluble protein from corn, as well as the albumins from egg or blood, fibrin from blood, and casein from cow's milk. Nitzescu<sup>5</sup> concluded on the basis of his work that persons suffering from pellagra have a ferment in their blood specific for zein. He found that the sera of all patients for whom a diagnosis of pellagra was certain, gave a positive Abderhalden reaction with zein. Twelve normal peasants on corn diet gave a negative reaction. In the hospital, of 14 patients with pellagra receiving bread, 10 gave negative reactions with gliadin from wheat and 4 indefinite reactions.

In addition to their proved antigenic properties, the vegetable proteins are highly satisfactory in work with the Abderhalden reaction because they do not tend to dialyze through animal membranes and in themselves give but a slight color reaction when boiled directly with ninhydrin. Moreover, when results discordant with specificity are obtained, the objection cannot be raised that the discrepancy is ascribable to blood cells or dialyzable substances remaining in the substrate. This latter feature suggested to von L. Flatow<sup>6</sup> the advantage of using casein in a similar study. He further emphasizes the fact that utilizing such pure materials as casein tends to eliminate the presence of proteins common to nearly all tissues, such, for example, as are present in connective tissue, whereby specificity might be clouded. Again, vegetable protein from the same sample can be employed repeatedly as substrate, this advantage enhancing the value of comparative results over those obtained by using samples from various organs—such as placenta—which have undergone autolytic digestion to different degrees. Bacterial infection is also less imminent in the

<sup>4</sup> Deutsch. med. Wchnschr., 1915, 41, p. 1151.

<sup>5</sup> Ibid., 1914, 40, p. 1614.

<sup>6</sup> München. med. Wchnschr., 1914, 61, 468.

vegetable proteins, which can be kept in dry condition in bottles, than in placental or other animal tissue preserved under toluene. In a word, these vegetable proteins in many cases represent as pure antigens as can be isolated, while most of the animal materials that have been studied represent mixtures of antigens.

#### EXPERIMENTAL WORK

Large vigorous male rabbits were almost exclusively employed. They were injected with the specific protein (usually intraperitoneally) in amounts and over a period of time as indicated in the tables. The intravenous method of immunization was not employed as being fraught with too much danger to the animals. Care had to be exercised not to inject too large a dose of the protein at one time, as fatal toxic action might result; closely repeated doses, given every day or every second day, of small amounts, varying from 0.1 to 0.2 gm., dissolved in a 0.1% NaOH solution were more efficient. Blood was obtained usually from the ear of the animal, sometimes directly from the heart.

The preparation of the serum and the method of dialysis as outlined by Abderhalden in the fourth edition of his "Abwehrfermente" were followed closely. Slight modifications, which would not depreciate the comparative value of the results, were introduced from time to time as seemed necessary to meet existing conditions. The serum was usually, but not invariably, free from hemoglobin. It was not examined spectroscopically. Hemoglobin-tinted serum was usually tested, however, as to its reactive power exactly as was the clear serum, for it was observed by us as well as by other workers that hemoglobin in the serum does not necessarily vitiate its usefulness. (Abderhalden insists that the sera must be absolutely free from hemoglobin even to spectroscope examinations.) Moreover, it is not always possible to obtain hemoglobin-free rabbit serum, even if the utmost precautions are exercised.

A very grievous difficulty exists in the use of rabbit serum in that it frequently contains dialyzable substances in amounts sufficient in themselves to give a strong positive reaction with ninhydrin. To eliminate this source of error part of the serum employed was predialyzed to running salt solution (0.85%) for periods ranging from 6 to 8 hours, before it was added to the substrates. This procedure usually served to remove all dialyzable substances and has been recommended by some investigators for use in all sera. With the object of reducing the amount of these dialyzable substances as much as possible, the rabbits were kept without food from 10 to 24 hours (a few, 36 hours) before drawing the blood.

The diffusion tubes of Schleicher and Shüll, No. 579a, were employed exclusively in the dialysis. They were frequently tested as outlined by Abderhalden. Each tube was numbered to correspond to a definite protein and was retained for that protein as exclusively as possible. Dialyzing flasks prepared for the Abderhalden reaction were found to be very serviceable as containers for the diffusion tubes.

In performing an experiment ordinarily 1 c.c. of serum was added to 0.1 gm. of the protein. Some variations were made in these amounts as are indicated later. Digestion continued for periods of time ranging from 16 to 24 hours, at a temperature of approximately 37 C., tho unavoidably some tests ran for a slightly longer period. The ninhydrin test was then made according to the

method laid down by Abderhalden. All experimental conditions were maintained as nearly uniform as possible at all times—this being deemed necessary to produce results of any comparative value. Falls<sup>7</sup> in a recent article especially emphasizes this feature. The depth of color obtained on boiling the dialyzed and ninhydrin solutions together is indicated by the symbols conventionally employed in immune reactions for that purpose; thus “+++” for a very deep blue-purple color, “++” for a less deep color, and so on, read after 30 minutes’ cooling. When the dialysate from the serum alone gave some reaction, the experiment was counted as positive only when the difference in depth of color between the dialysate of the serum, and that of the protein and serum was marked enough to merit such a decision beyond any possible doubt. If the two dialysates showed a reaction of about the same degree the experiment was considered questionable. This procedure has support in the work of Pearce and Williams,<sup>8</sup> and in the directions of Abderhalden. There is no apparent reason why the view is not tenable.

The sera of many of the rabbits were tested for the presence of reacting substances against the individual proteins, before any immunization had taken place. At no time did a reaction develop after the period of incubation designated, which could not be ascribed to dialyzable substances present in the normal serum itself: that is, there was no apparent interaction between the normal serum and protein substrate. Because of these negative findings, it was not deemed necessary to subject the sera of all the rabbits to this test. After immunization it was not always possible to procure sufficient serum at any given time to run the full quota of tests; hence some irregularities may appear in the number of reactions obtained from the various proteins. No attempt was made at this stage of the work to determine how quickly antibodies to these various antigens appeared in the animals’ sera, which could be detected by the dialysis method, nor to determine what the nature of these antibodies is. The serum was usually obtained from 3 to 4 days subsequent to the last previous injection of protein.

The proteins used as antigens were (A) edestin, a globulin from the hemp-seed (*Cannabis sativa*); (B) a globulin from the squash seed (*Cucurbita maxima*); (C) gliadin, an alcohol-soluble protein from wheat (*Triticum vulgare*); (D) hordein, an alcohol-soluble protein from barley (*Hordeum vulgare*); (E) pea legumin, a globulin from the pea (*Pisum sativum*); (F) phaseolin, a globulin from the adzuki bean (*Phaseolus radiatus*); (G) glycinin, a globulin from the soy bean (*Sojahi spida*); (H) fresh egg white. The nature and results of experiments are herewith recorded.

#### A. WITH EDESTIN AS ANTIGEN

*Rabbit 1.*—Injected with a total of 4.5 gm. of edestin from March 8 to June 26, 1915. Blood was drawn and serum obtained April 12, 21, 30, May 12, 24, 1915. The experiments of April 12, May 12, and May 24 were questionable and are discarded because of the presence of sufficient dialyzable substances in the serum to obscure the definiteness of the ninhydrin reaction in differentiating between the serum and protein, and the serum alone. In this series as in the subsequent ones, the Arabic figures designate the number of a given experiment for a particular rabbit, while the Roman numerals indicate the position of the same experiment in the series for the given protein antigen.

<sup>7</sup> Jour. Am. Med. Assn., 1914, 63, p. 1172.

<sup>8</sup> Jour. Infect. Dis., 1914, 14, p. 351.

TABLE 1  
RESULTS OF ABDERHALDEN TEST EMPLOYING EDESTIN AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, I	April 21	22	0.01 edestin.....	1 1*	+ Trace
2, II	April 30	22	0.01 edestin.....	1	++
			0.01 gliadin (wheat).....	1	Strong trace
				1*	Strong trace

\* Control.

*Rabbit 2.*—Injected with a total of 4.2 gm. of edestin from April 12 to May 28, 1915. Blood was drawn and serum obtained April 17, 26, 29, May 20, and 29. The experiments of April 17 and May 29 were questionable and are discarded because of the presence of sufficient dialyzable substances in the serum to obscure the definiteness of the ninhydrin reaction in differentiating between the serum and protein, and the serum alone.

TABLE 2  
RESULTS OF ABDERHALDEN TEST EMPLOYING EDESTIN AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, III	April 27	23	0.01 edestin.....	1 1*	++ Faint trace
2, IV	April 29	24	0.01 edestin.....	1	++
			0.01 gliadin (wheat).....	1*	Faint trace
				0.75 0.75*	Faint trace Faint trace <sup>2</sup>
3, V	May 1	23	0.01 edestin.....	1	+ Strong
			0.01 globulin (squash seed).....	1	0
			0.01 hordein.....	1	0
			0.01 legumin (pea).....	1	0
			0.01 legumin (horse bean).....	1	0
			0.01 globulin (black walnut).....	1*	0

\* Control.

*Rabbit 3.*—Injected with a total of 2 gm. of edestin from Oct. 15, to Oct. 27, 1915. Blood was drawn and serum obtained Oct. 26. Only one experiment was performed on this animal, as it died from the toxic effects of an over dose of the protein on Oct. 28. Blood was drawn immediately after death from the heart. This result is presented with some hesitancy on account of the extreme hemoglobin content of the sera, probably due to the fact that the blood had stood in the icebox over night.

TABLE 3  
RESULTS OF ABDERHALDEN TEST EMPLOYING EDESTIN AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, VI	Oct. 27	23	0.01 edestin.....	1	++
			0.01 excelsin.....	1	0
				1*	0

\* Control.

*Rabbit 4.*—Injected with a total of 3.1 gm. of edestin from Nov. 1 to Dec. 6, 1915. Blood was drawn and serum obtained Nov. 29 and Dec. 7. The experiment of Dec. 7 is discarded as the rabbit at that time appeared ill from "snuffles."

TABLE 4  
RESULTS OF ABDERHALDEN TEST EMPLOYING EDESTIN AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, c.c.	Reaction
I, VII	Nov. 29	19	0.1 edestin.....	1	Trace
			0.1 globulin (squash seed).....	1	Trace
			0.1 excelsin.....	1	+ (?)
			0.1 gliadin (wheat).....	1	++
			0.1 legumin (pea).....	1	Trace
				1*	Trace

\* Control.

*Rabbit 5.*—Injected with a total of 1.7 gm. of edestin from Jan. 17 to March 13, 1916. Blood was drawn and serum obtained Feb. 11 and 22, and March 3. The serum of March 3 was predialyzed to running salt solution (0.85%) for 8½ hours.

TABLE 5  
RESULTS OF ABDERHALDEN TEST EMPLOYING EDESTIN AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, VIII	Feb. 11	20	0.1 edestin.....	1	++
			0.05 edestin.....	1.5	++ Strong
				1.5*	Trace
			0.1 globulin (squash seed).....	1	Trace
			0.1 excelsin.....	1	+
			0.1 gliadin (wheat).....	1	Strong trace
			0.1 gliadin (rye).....	1	Trace
			0.1 legumin (pea).....	1	+
			0.1 hordein.....	1	0
			0.1 egg white, dry.....	1	0
				1*	Trace
2, IX	Feb. 22	18	0.1 edestin.....	1	+ Strong
			0.2 edestin.....	1.5	+ Strong
				1.5*	Trace
			0.1 globulin (squash seed).....	1	0
			0.05 excelsin.....	1	0
			0.1 gliadin (wheat).....	1	+
			0.1 legumin (pea).....	1	+
			0.1 hordein.....	1	0
			0.1 egg white, dry.....	1	0
				1*	0
3, X	Mar. 3	18	0.1 edestin.....	1.5	+
			0.2 edestin.....	1.5*	0
				1	Trace ?
			0.1 globulin (squash seed).....	1	0
			0.1 gliadin (wheat).....	1	0
			0.1 legumin (pea).....	1	0
			0.1 hordein.....	1	0
			0.1 egg white, dry.....	1	0
				1*	0

\* Control.

*Rabbit 6.*—Injected with a total of 1.7 gm. of edestin from Jan. 17 to March 13, 1916. Blood was drawn and serum obtained Feb. 11 and 22, and March 3, 1916. The sera of Feb. 11 and 22 were predialyzed to running salt solution (0.85%) for 7 hours.

TABLE 6  
RESULTS OF ABDERHALDEN TEST EMPLOYING EDESTIN AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, XI	Feb. 11	23	0.1 edestin.....	1	Strong trace
			0.2 edestin.....	1.5	+
			0.05 edestin.....	1.5	0
				1.5*	0
			0.1 globulin (squash seed).....	1	+
			0.1 excelsin.....	1	0
			0.1 gliadin (wheat).....	1	Strong trace
			0.1 gliadin (rye).....	1	+
			0.1 legumin (pea).....	1	Trace ?
			0.1 hordein.....	1	+
			0.1 egg white, dry.....	1	0
				1*	0
2, XII	Feb. 22	17	0.1 edestin.....	1	++
			0.2 edestin.....	1.5	++
				1.5*	0
			0.1 globulin (squash seed).....	1	0
			0.05 excelsin.....	1	0
			0.1 gliadin (wheat).....	1	0
			0.1 gliadin (rye).....	1	+
			0.1 legumin (pea).....	1	0
			0.1 hordein.....	1	0
			0.1 prolamin (oat).....	1	0
			0.1 egg white, dry.....	1	0
				1*	0
2, XIII	Mar. 3	18	0.1 edestin.....	1.5	0
				1.5*	0
			0.2 edestin.....	1	++
			0.1 globulin (squash seed).....	1	0
			0.05 excelsin.....	1	+
			0.1 gliadin (wheat).....	1	Trace
			0.1 legumin (pea).....	1	0
			0.1 hordein.....	1	0
			0.1 egg white, dry.....	1	0
			0.1 prolamin (oat).....	1	+
			0.1 globulin (castor bean).....	1	0
			0.1 globulin (cocoanut).....	1	+
			0.1 alcohol-sol. protein from millet...	1	0
				1*	0

\* Control.

A summary of the experiments with edestin is given in Table 7.

TABLE 7

SUMMARY OF THE RESULTS OF ABDERHALDEN TESTS EMPLOYING EDESTIN AS ANTIGEN

Protein	Number of Tests	"++ Strong" or "++"	"+ Strong" or "+"	Strong Trace	Trace or Faint Trace	Questionable	No Reactions
Edestin.....	18	9	6	1	0	1	1
Globulin (squash seed).....	6	0	1	0	0	0	5
Excelsin.....	7	0	2	0	0	1	4
Gliadin (wheat).....	7	1	1	2	1	0	2
Gliadin (rye).....	2	0	2	0	0	0	0
Legumin (pea).....	7	0	2	0	0	1	4
Hordein.....	7	0	1	0	0	0	6
Prolamin (oat).....	2	0	1	0	0	0	1
Egg white, dry.....	6	0	0	0	0	0	6
Globulin (castor bean).....	1	0	0	0	0	0	1
Cocoonut protein.....	1	0	1	0	0	0	0
Alcohol soluble protein from millet	1	0	0	0	0	0	1
Legumin (horse bean).....	1	0	0	0	0	0	1
Globulin (black walnut).....	1	0	0	0	0	0	1

#### B. WITH SQUASH-SEED GLOBULIN AS ANTIGEN

*Rabbit 1.*—Injected with a total of 5.8 gm. of squash-seed globulin from Oct. 16 to Dec. 10, 1915. Blood was drawn and serum obtained Nov. 2 and 27, and Dec. 10. The experiment of Nov. 2 was questionable, as the animal's blood apparently contained an excess of dialyzable substances, in spite of previous starvation. However, the differences in color reaction were sufficient to warrant its being included in the tabulations.

TABLE 8

RESULTS OF ABDERHALDEN TEST EMPLOYING SQUASH-SEED GLOBULIN AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, I	Nov. 2	23	0.01 globulin (squash seed).....	1.5	+ Strong
			0.01 excelsin.....	1.5	Strong trace
				1.5*	Strong trace
2, II	Nov. 27	24	0.1 globulin (squash seed).....	1	+
			0.1 excelsin.....	1	++
			0.1 gliadin (wheat).....	1	Strong trace
			0.1 legumin (pea).....	1	0
			0.1 edestin.....	1	0
				1*	0
3, III	Dec. 10	17	0.1 globulin (squash seed).....	1	0
			0.1 globulin (squash seed).....	1	0
			0.1 excelsin.....	1	0
			0.1 gliadin (wheat).....	1	0
			0.1 gliadin (rye).....	1	0
			0.1 legumin (pea).....	1	+
			0.1 edestin.....	1	0
			0.1 hordein.....	1	Strong trace
			0.1 prolamin (oat).....	0.75	Trace
				1*	Trace

\* Control.

*Rabbit 2.*—Injected with a total of 7.8 gm. of squash-seed globulin from Oct. 22, 1915, to March 13, 1916. Blood was drawn and serum obtained Nov. 23, Dec. 10, 1915, Jan. 8, 21, Feb. 24, and March 7, 1916. The experiment of Jan. 8 is questionable on account of strong reactions obtained with serum alone, due apparently to an excess of dialyzable substances in the serum. The sera of Jan. 21 and Feb. 24 were predialyzed to running salt solution (0.85%) for 6 hours.

TABLE 9  
RESULTS OF ABDERHALDEN TEST EMPLOYING SQUASH-SEED GLOBULIN AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, IV	Nov. 23	19	0.01 globulin (squash seed).....	1	+
			0.01 excelsin.....	1	Strong trace
			0.01 edestin.....	1	0
				1*	0
2, V	Dec. 10	17	0.01 globulin (squash seed).....	1	Strong trace
			0.1 excelsin.....	1	+
			0.1 gliadin (wheat).....	1	+
			0.1 gliadin (rye).....	1	0
			0.1 legumin (pea).....	1	Trace
			0.1 edestin.....	1	0
			0.1 hordein.....	1	Strong trace
			0.1 fresh egg white (concentrated)....	1	Trace
3, VI	Jan. 21	18		1*	0
			0.1 globulin (squash seed).....	1	0
			0.1 excelsin.....	1	Strong trace
			0.1 gliadin (wheat).....	1	+
			0.1 gliadin (rye).....	1	Strong trace
			0.1 legumin (pea).....	1	+
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
4, VII*	Jan. 21	18		1*	0
			0.1 globulin (squash seed).....	1	0
			0.1 excelsin.....	1	0
			0.1 gliadin (wheat).....	1	+
			0.1 legumin (pea).....	1	0
			0.1 hordein.....	1	0
5, VIII	Feb. 24	17		1*	0
			0.1 globulin (squash seed).....	1	0
			0.5 excelsin.....	1	0
			0.1 gliadin (wheat).....	1	Strong trace
			0.1 gliadin (rye).....	1	0
			0.1 legumin (pea).....	1	Strong trace
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
6, IX	Mar. 7	18	0.1 egg white, dry.....	1	0
				1	0
			0.1 globulin (squash seed).....	1	++
			0.2 globulin (squash seed).....	1.5	0
				1.5*	0
			0.1 excelsin.....	1	0
			0.1 gliadin (wheat).....	1	Trace
			0.1 legumin (pea).....	1	++
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
				1*	0
				1*	0

\* Control.

† The serum in Experiment VII is of the same sample as that in Experiment VI, but it was predialyzed for 6 hours before use.

*Rabbit 3.*—Injected with a total of 2.25 gm. squash-seed globulin from Jan. 12 to March 13, 1916. Blood was drawn and serum obtained Feb. 24 and March 7. The serum of March 7 was predialyzed to running salt solution (0.85%) for 7 hours.

TABLE 10  
RESULTS OF ABDERHALDEN TEST EMPLOYING SQUASH-SEED GLOBULIN AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, X	Feb. 24	19	0.1 globulin (squash seed).....	1	+
			0.2 globulin (squash seed).....	1.5	++
				1.5*	0
			0.05 excelsin.....	1	0
			0.1 gliadin (wheat).....	1	0
			0.1 gliadin (rye).....	1	0
			0.1 legumin (pea).....	1	0
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
			0.1 prolamín (oat).....	1	0
			0.1 egg white, dry.....	1	0
			0.1 globulin (castor bean).....	1	+
			0.1 cocoanut protein.....	1	0
				1*	0
2, XI	Mar. 7	18	0.1 globulin (squash seed).....	1	Trace
			0.2 globulin (squash seed).....	1	Trace
				1.5*	0
			0.1 excelsin.....	1	0
			0.1 gliadin (wheat).....	1	Trace
			0.1 legumin (pea).....	1	Trace
			0.1 edestin.....	1	0

\* Control.

A summary of the experiments with squash-seed globulin is given in Table 11.

TABLE 11  
RESULTS OF ABDERHALDEN TEST EMPLOYING SQUASH-SEED GLOBULIN AS ANTIGEN

Protein	Number of Tests	"++ Strong" or "++"	"+ Strong" or "+"	Strong Trace	Trace or Faint Trace	Questionable	No Reactions
Globulin (squash seed).....	15	2	4	1	2	0	6
Excelsin.....	10	1	1	2	0	0	6
Gliadin (wheat).....	9	0	3	2	2	0	2
Gliadin (rye).....	5	0	0	1	0	0	4
Legumin (pea).....	9	1	2	2	2	0	3
Edestin.....	9	0	0	0	0	0	0
Hordein.....	7	0	0	2	0	0	5
Egg white, dry.....	3	0	0	0	1	0	2
Prolamin (oat).....	2	0	0	0	1	0	1
Globulin (castor bean).....	1	0	1	0	0	0	0
Cocoanut protein.....	1	0	0	0	0	0	1

### C. WITH GLIADIN FROM WHEAT AS ANTIGEN

*Rabbit 1.*—Injected with a total of 1.6 gm. gliadin from wheat from Oct. 19 to Oct. 22, 1915. Blood was drawn and serum obtained Oct. 26. The rabbit died shortly afterward as the result of wound infection.

TABLE 12  
RESULTS OF ABDERHALDEN TEST EMPLOYING GLIADIN (WHEAT) AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, I	Oct. 26	24	0.01 gliadin (wheat).....	1	+
			0.01 gliadin (rye).....	1	+ Strong
			0.1 edestin.....	1	+
			0.1 prolamín (oat).....	1	0
				1*	Faint trace

\* Control.

*Rabbit 2.*—Injected with a total of 5.15 gm. gliadin from wheat from Oct. 30, 1915, to March 3, 1916. Blood was drawn and serum obtained Nov. 28, Dec. 8, 1915, Jan. 21, Feb. 17, 25, and March 6, 1916. Part of the serum obtained Jan. 21, as well as that of Feb. 17, 25, and March 2, was predialyzed to running salt solution (0.85%) for about 6 hours.

TABLE 13  
RESULTS OF ABDERHALDEN TEST EMPLOYING GLIADIN (WHEAT) AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, II	Nov. 28	18	0.1 gliadin (wheat).....	1	++
			0.1 gliadin (rye).....	1	0
			0.1 legumin (pea).....	1	+
			0.1 edestin.....	1	0
			0.1 hordein.....	1	Trace (?)
			0.1 globulin (squash seed).....	1	+
			0.1 prolamin (oat).....	1	Strong trace
				1*	0
2, III	Dec. 8	18	0.1 gliadin (wheat).....	1	+
			0.1 gliadin (wheat).....	1.5	++
				1.5*	Trace
			0.1 gliadin (rye).....	1	0
			0.1 legumin (pea).....	1	+
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	+
			0.1 prolamin (oat).....	1	Trace (?)
			0.1 alcohol-sol. protein from millet...	1	0
				1*	0
3, IV	Jan. 21	16	0.1 gliadin (wheat).....	1	+
			0.1 legumin (pea).....	1	Strong trace
			0.1 edestin.....	1	+
			0.1 hordein.....	1	Strong trace
			0.1 globulin (squash seed).....	1	Strong trace
				1*	0
4, V†	Jan. 21	17	0.1 gliadin (wheat).....	1	+
			0.1 legumin (pea).....	1	0
			0.1 edestin.....	1	Faint trace
			0.1 hordein.....	1	Faint trace
			0.1 globulin (squash seed).....	1	Faint trace
				1*	0
5, VI	Feb. 17	17	0.1 gliadin (wheat).....	1	+
			0.2 gliadin (wheat).....	1.5	++
				1.5*	0
			0.1 gliadin (rye).....	1	+
			0.1 legumin (pea).....	1	Strong trace
			0.1 edestin.....	1	Strong trace
			0.1 hordein.....	1	Faint trace
			0.1 globulin (squash seed).....	1	Strong trace
			0.1 prolamin (oat).....	1	Strong trace
			0.1 egg white, dry.....	1	Faint trace
				1*	0
6, VII	Feb. 25	18	0.1 gliadin (wheat).....	1	+
			0.2 gliadin (wheat).....	1.5	+
				1.5*	0
			0.1 gliadin (rye).....	1	0
			0.1 legumin (pea).....	1	Faint trace
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	0
			0.1 prolamin (oat).....	1	0
			0.1 egg white, dry.....	1	++ ‡
				1	0

\* Control.

† The serum in Experiment V was of the same sample as that in Experiment IV, but it was predialyzed before use. The reactions are weaker than in the latter experiment, but they nevertheless correspond fairly well.

‡ This inexplicable reaction is probably the result of faulty technic.

TABLE 13—*Continued*

RESULTS OF ABDERHALDEN TEST EMPLOYING GLIADIN (WHEAT) AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
7, VIII	Mar. 6	18	0.1 gliadin (wheat).....	1	+
			0.2 gliadin (wheat).....	1.5	++
			0.1 gliadin (rye).....	1	+
			0.1 legumin (pea).....	1	0
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	0
			0.1 prolamin (oat).....	1	+
			0.1 egg white, dry.....	1	0
				1*	Trace

\* Control.

*Rabbit 3.*—Injected with a total of 1.75 gm. of gliadin from wheat from Jan. 13 to March 3, 1916. Blood was drawn and serum obtained on Feb. 14, 25, and March 6.

TABLE 14

RESULTS OF ABDERHALDEN TEST EMPLOYING GLIADIN (WHEAT) AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, IX	Feb. 17	18	0.1 gliadin (wheat).....	1	+
			0.2 gliadin (wheat).....	1.5	++
				1.5*	Faint trace
			0.1 gliadin (rye).....	1	+
			0.1 legumin (pea).....	1	Strong trace
			0.1 edestin.....	1	Faint trace
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	0
			0.1 egg white, dry.....	1	0
				1*	Error
2, X	Feb. 25	18	0.1 gliadin (wheat).....	1	++
			0.2 gliadin (wheat).....	1.5	++
				1.5*	Faint trace
			0.1 gliadin (rye).....	1	+
			0.1 legumin (pea).....	1	Faint trace
			0.1 edestin.....	1	Faint trace
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	0
			0.1 prolamin (oat).....	0.75	0
			0.1 egg white, dry.....	1	0
				1*	0
3, XI	Mar. 6	18	0.1 gliadin (wheat).....	1	+
			0.1 gliadin (rye).....	1	+
			0.1 legumin (pea).....	1	0
			0.1 edestin.....	1	Trace (?)
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	0
			0.1 prolamin (oat).....	1	0
				1*	0

\* Control.

A summary of the experiments with gliadin (wheat) is given in Table 15.

TABLE 15

Protein	Number of Tests	"++ Strong" or "++"	"+ Strong" or "+"	Strong Trace	Trace or Faint Trace	Questionable	No Reaction
Gliadin (wheat).....	16	6	10	0	0	0	0
Gliadin (rye).....	9	0	6	0	0	0	3
Legumin (pea).....	10	0	2	3	2	0	3
Edestin.....	11	0	2	1	2	2	4
Hordein.....	10	0	0	1	2	1	6
Globulin (squash seed).....	10	0	2	2	1	0	5
Prolamin (oat).....	8	0	2	2	0	1†	3
Egg white, dry.....	5	0	0	0	1	1	3
Alcohol-soluble protein from millet	1	0	0	0	0	0	1

† See note (†) Table 13.

#### D. WITH HORDEIN AS ANTIGEN

*Rabbit 1.*—Injected with a total of 1.6 gm. of hordein from Jan. 18 to March 13, 1916. Blood was drawn and serum obtained Feb. 14, 29, and March 17. All the sera were predialyzed to running salt solution (0.85%) for 7, 7½, and 7½ hours respectively.

TABLE 16

RESULTS OF ABDERHALDEN TEST EMPLOYING HORDEIN AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, I	Feb. 14	19	0.1 hordein.....	1	+
			0.2 hordein.....	1.5	0
				1.5*	0
			0.1 globulin (squash seed).....	1	0
			0.1 gliadin (wheat).....	1	+
			0.1 gliadin (rye).....	1	0
			0.1 edestin.....	1	0
			0.1 legumin (pea).....	1	0
			0.1 prolamin (oat).....	1	+
			0.1 egg white, dry.....	1	+
				1*	+(?)†
2, II	Feb. 29	18	0.1 hordein.....	1	+
			0.2 hordein.....	1.5	+
				1.5*	0
			0.1 globulin (squash seed).....	1	+
			0.1 gliadin (wheat).....	1	+
			0.1 gliadin (rye).....	1	+
			0.1 edestin.....	1	+
			0.1 legumin (pea).....	1	Faint trace
			0.1 prolamin (oat).....	1	+
			0.1 egg white, dry.....	1	+
				1*	0
3, III	Mar. 17	20	0.1 hordein.....	1	+
			0.1 hordein.....	1	+
			0.1 globulin (squash seed).....	1	+
			0.1 gliadin (wheat).....	1	Strong trace
			0.1 gliadin (rye).....	1	0
			0.1 edestin.....	1	Trace (?)
			0.1 legumin (pea).....	1	0
				1*	0

\* Control.

† Probably an error, since 1.5 c.c. serum (as control) in the same experiment was negative.

*Rabbit 2.*—Injected with a total of 1.5 gm. hordein from Jan. 18 to March 17, 1916. Blood was drawn and serum obtained Feb. 14, 29, and March 17. None of the sera was predialyzed. The reaction of the experiment of March 17 was questionable and is discarded. The serum apparently contained too many dialyzable substances which in themselves gave a strong reaction.

TABLE 17  
RESULTS OF ABDERHALDEN TEST EMPLOYING HORDEIN AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, IV	Feb. 4	17	0.1 hordein.....	1	+
			0.2 hordein.....	1.5	+
				1.5*	0
			0.1 globulin (squash seed).....	1	Trace
			0.1 gliadin (wheat).....	1	Strong trace
			0.1 gliadin (rye).....	1	++
			0.1 edestin.....	1	+
			0.1 legumin (pea).....	1	Strong trace
2, V	Feb. 29	18		1*	0
			0.1 hordein.....	1	++
			0.2 hordein.....	1.5	0
				1.5*	0
			0.1 globulin (squash seed).....	1	++
			0.1 gliadin (wheat).....	1	0
			0.1 edestin.....	1	0
			0.1 legumin (pea).....	1	Faint (?)
			0.1 prolamin (oat).....	1	0
			0.1 egg white, dry.....	1	0
				1*	0

\* Control.

A summary of the experiments with hordein is given in Table 18.

TABLE 18  
SUMMARY OF RESULTS OF ABDERHALDEN TESTS EMPLOYING HORDEIN AS ANTIGEN

Protein	Number of Tests	"++ Strong" or "++"	"+" Strong" or "+"	Strong Trace	Trace or Faint Trace	Ques- tion- able	No Reac- tions
Hordein.....	10	1	7	0	0	0	2
Globulin (squash seed).....	5	1	2	0	1	0	1
Gliadin (wheat).....	5	0	2	2	0	0	1
Gliadin (rye).....	4	1	1	0	0	0	2
Edestin.....	5	0	2	0	1	0	2
Legumin (pea).....	5	0	0	1	1	1	2
Prolamin (oat).....	3	0	2	0	0	0	1
Egg white, dry.....	3	0	2	0	0	0	1

#### E. WITH PEA LEGUMIN AS ANTIGEN

*Rabbit 1.*—Injected with a total of 4.5 gm. pea legumin from Oct. 22 to Dec. 6, 1915. Blood was drawn and serum obtained Nov. 10, 27, and Dec. 6. The experiment of Nov. 10 was entirely negative, no reactive power apparently having been developed in the serum.

TABLE 19  
RESULTS OF ABDERHALDEN TEST EMPLOYING PEA LEGUMIN AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, I	Nov. 27	18	0.01 legumin (pea).....	1	Strong trace
			0.01 edestin.....	1	Strong trace
			0.01 globulin (squash seed).....	1	+
			0.01 gliadin (wheat).....	1	++
				1*	Trace
2, II	Dec. 21	16	0.1 legumin (pea).....	1	++
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	Trace (?)
			0.1 gliadin (wheat).....	1	+
			0.1 legumin (horse bean).....	1	Strong trace
				1*	0

\* Control

*Rabbit 2.*—Injected with a total of 1.9 gm. pea legumin from Jan. 18 to April 9, 1916. Blood was drawn and serum obtained Feb. 8, March 1, 16, April 9, and 12. The serum of Feb. 8 and 16, and of April 9 and 12, were predialyzed to running salt solution (0.85%) for 6, 6, 7, and 6 hours, respectively, before use.

TABLE 20  
RESULTS OF ABDERHALDEN TEST EMPLOYING PEA LEGUMIN AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, e.c.	Reaction
1, III	Feb. 8	18	0.1 legumin (pea).....	1	+
			0.5 legumin (pea).....	1.5	+
				1.5*	0
			0.1 edestin.....	1	0
			0.1 hordein.....	1	+
			0.1 globulin (squash seed).....	1	++
			0.1 gliadin (wheat).....	1	+
				1*	0
2, IV	Feb. 15	18	0.1 legumin (pea).....	1	++
			0.2 legumin (pea).....	1.5	++
				1.5*	Strong trace
			0.1 edestin.....	1	+
			0.1 hordein.....	1	Faint trace ?
			0.1 globulin (squash seed).....	1	+
			0.1 gliadin (wheat).....	1	+
			0.1 egg white, dry.....	1	0
			0.1 glycinin (soy bean).....	1	Trace
			0.1 legumin (horse bean).....	1	Trace
			0.1 legumin (vetch).....	1	Trace
			0.1 vicilin (pea).....	1	Trace
				1*	0
3, V	Mar. 1	18	0.1 legumin (pea).....	1	+
			0.2 legumin (pea).....	1.5	++
				1.5*	Strong trace
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	0
			0.1 gliadin (wheat).....	1	0
			0.1 prolamins (oat).....	1	Trace
			0.1 egg white, dry.....	1	0
			0.1 legumin (horse bean).....	1	Trace
				1*	0
4, VI	Mar. 16	18	0.1 legumin (pea).....	1	
			0.1 phaseolin (adzuki bean).....	1	++
			0.1 glycinin (soy bean).....	1	Trace
			0.1 legumin (horse bean).....	1	+
			0.1 legumin (vetch).....	1	++
			0.1 vicilin (pea).....	1	+
			0.1 legumin (lentil).....	1	+
			0.1 viginin (cow pea).....	1	++
				1*	0
5, VII	April 12	18	0.1 legumin (pea).....	1	+
			0.1 phaseolin (adzuki bean).....	1	Faint trace
			0.1 glycinin (soy bean).....	1	0
			0.1 legumin (vetch).....	1	0
			0.1 vicilin (pea).....	1	Faint trace
			0.1 legumin (lentil).....	1	Faint trace
				1*	Faint trace
6, VIII	April 12	18	0.1 legumin (pea).....	1	++
			0.1 phaseolin (adzuki bean).....	1	0
			0.1 glycinin (soy bean).....	1	0
			0.1 legumin (vetch).....	1	0
			0.1 vicilin (pea).....	1	+
			0.1 legumin (lentil).....	1	0
			0.1 viginin (cow pea).....	1	0
				1*	0

\* Control.

*Rabbit 3.*—Injected with a total of 1.9 gm. pea legumin from Jan. 10 to April 9, 1916. Blood was drawn and serum obtained Feb. 8, 15, March 1, 16, April 9, and 12. The sera of March 1, March 16, April 9, and April 12 were predialyzed to running salt solution (0.85%) for 5, 8, 7, and 6 hours, respectively. The experiment of April 12 was questionable and is discarded for reasons previously indicated.

TABLE 21  
RESULTS OF ABDERHALDEN TEST EMPLOYING PEA LEGUMIN AS ANTIGEN

Test	Date	Ineuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, IX	Feb. 8	18	0.1 legumin (pea).....	1	+
			0.1 edestin.....	1	+ Strong
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	0
			0.1 gliadin (wheat).....	1	0
			0.1 glycinin (soy bean).....	1	0
			0.1 legumin (horse bean).....	1	0
			0.1 legumin (vetch).....	1	0
			0.1 vicilin (pea).....	1	0
			0.1 legumin (lentil).....	1	0
				1*	0
2, X	Feb. 15	17	0.1 legumin (pea).....	1	++
			0.2 legumin (pea).....	1.5	++
				1.5*	0
			0.1 edestin.....	1	0
			0.1 hordein.....	1	Strong trace
			0.1 globulin (squash seed).....	1	0
			0.1 gliadin (wheat).....	1	Strong trace
			0.1 egg white, dry.....	1	Trace
			0.1 glycinin (soy bean).....	1	Strong trace
			0.1 legumin (horse bean).....	1	Strong trace
			0.1 legumin (vetch).....	1	Strong trace
			0.1 vicilin (pea).....	1*	0
3, XI	Mar. 1	18	0.1 legumin (pea).....	1	Trace
			0.2 legumin (pea).....	1.5	++
				1.5*	0
			0.1 edestin.....	1	0
			0.1 hordein.....	1	0
			0.1 globulin (squash seed).....	1	0
			0.1 gliadin (wheat).....	1	++
			0.1 prolamins (oat).....	1	0
			0.1 egg white, dry.....	1	0
				1*	0
4, XII	Mar. 16	18	0.1 legumin (pea).....	1	+
			0.1 phaseolin (adzuki bean).....	1	Trace (?)
			0.1 glycinin (soy bean).....	1	Trace (?)
			0.1 legumin (horse bean).....	1	+
			0.1 legumin (vetch).....	1	Trace (?)
			0.1 vicilin (pea).....	1	+
			0.1 legumin (lentil).....	1	+
			0.1 viginin (cow pea).....	1	+
			0.1 globulin (castor bean).....	1	++
				1*	0
5, XIII	April 9	19	0.1 legumin (pea).....	1	+
			0.1 phaseolin (adzuki bean).....	1	Trace
			0.1 glycinin (soy bean).....	1	Trace
			0.1 legumin (vetch).....	1	Trace
			0.1 vicilin (pea).....	1	++
			0.1 legumin (lentil).....	1	Trace
			0.1 viginin (cow pea).....	1	Trace
				1*	0

\* Control

A summary of the experiments with pea legumin is given in Table 22.

TABLE 22  
SUMMARY OF RESULTS OF ABDERHALDEN TESTS EMPLOYING PEA LEGUMIN AS ANTIGEN

Protein	Number of Tests	"++ Strong or ++"	" + Strong" or +"	Strong Trace	Trace or Faint Trace	Ques- tion- able	No Reac- tion
Legumin (pea).....	18	8	8	1	1	0	0
Edestin.....	8	0	2	1	0	0	5
Hordein.....	7	0	1	1	0	1	4
Globulin (squash seed).....	8	1	2	0	0	1	4
Gliadin (wheat).....	8	2	3	1	0	0	2
Prolamin (oat).....	2	0	0	0	1	0	1
Egg white, dry.....	3	0	0	0	1	0	2
Phaseolin (adzuki bean).....	5	1	0	0	2	1	1
Glycinin (soy bean).....	8	0	0	1	3	1	3
Legumin (horse bean).....	6	0	2	2	1	0	1
Legumin (vetch).....	8	1	0	1	2	1	3
Vicilin (pea).....	8	1	3	1	2	0	1
Legumin (lentil).....	6	0	2	0	2	0	2
Vignin (cow pea).....	4	1	1	0	1	0	1
Globulin (castor bean).....	1	1	0	0	0	0	0

F. WITH PHASEOLIN FROM ADZUKI BEAN AS ANTIGEN

*Rabbit 1.*—Injected with a total of 0.7 gm. phaseolin from March 15 to April 8, 1916. Blood was drawn and serum obtained March 29, April 8, and 11. The sera were all predialyzed to running salt solution (0.85%) for 8, 10, and 6 hours, respectively.

TABLE 23  
RESULTS OF ABDERHALDEN TESTS EMPLOYING PHASEOLIN (ADZUKI BEAN) AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, I	Mar. 29	22	0.1 phaseolin.....	1	+
			0.1 glycinin (soy bean).....	1	0
			0.1 legumin (pea).....	1	0
			0.1 legumin (vetch).....	1	Strong trace
			0.1 vicilin (pea).....	1	++
			0.1 legumin (lentil).....	1	Trace
			0.1 vignin (cow pea).....	1	Trace
			1*	0	
2, II	April 8	19	0.1 phaseolin.....	1	+
			0.1 glycinin (soy bean).....	1	0
			0.1 legumin (pea).....	1	0
			0.1 legumin (vetch).....	1	Trace
			0.1 vicilin (pea).....	1	Trace
			0.1 legumin (lentil).....	1	0
			0.1 vignin (cow pea).....	1	0
			1*	0	
3, III	April 11	20	0.1 phaseolin.....	1	Strong trace
			0.1 glycinin (soy bean).....	1	Strong trace
			0.1 legumin (pea).....	1	Strong trace
			0.1 legumin (vetch).....	1	+
			0.1 vicilin (pea).....	1	+
			0.1 legumin (lentil).....	1	Trace
			0.1 vignin (cow pea).....	1	+
			1*	Trace	

\* Control.

*Rabbit 2.*—Injected with a total of 0.7 gm. phaseolin from March 15 to April 8, 1916. Blood was drawn and serum obtained March 29, April 8, and 11. The sera were all predialyzed to running salt solution (0.85%) for 8, 10, and 6 hours, respectively.

TABLE 24  
RESULTS OF ABDERHALDEN TESTS EMPLOYING PHASEOLIN (ADZUKI BEAN) AS ANTIGEN

Test	Date	Incubation, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, IV	Mar. 29	22	0.1 phaseolin.....	1	Trace
			0.1 glycinin (soy bean).....	1	Trace
			0.1 legumin (pea).....	1	+
			0.1 legumin (vetch).....	1	Trace
			0.1 vicilin (pea).....	1	Trace
			0.1 legumin (lentil).....	1	+
			0.1 viginin (cow pea).....	1*	0
2, V	April 9	19	0.1 phaseolin.....	1	+ Strong
			0.1 glycinin (soy bean).....	1	Faint trace
			0.1 legumin (pea).....	1	Faint trace
			0.1 legumin (vetch).....	1	Faint trace
			0.1 vicilin (pea).....	1	Faint trace
			0.1 legumin (lentil).....	1	Faint trace
3, VI	April 11	20		1*	0
			0.1 phaseolin.....	1	Strong trace
			0.1 glycinin (soy bean).....	1	Strong trace
			0.1 legumin (pea).....	1	Strong trace
			0.1 legumin (vetch).....	1	+
			0.1 vicilin (pea).....	1	+
			0.1 legumin (lentil).....	1	Faint trace
			0.1 viginin (cow pea).....	1	Faint trace
				1*	0

\* Control.

A summary of the experiments with phaseolin is given in Table 25.

TABLE 25  
SUMMARY OF RESULTS OF ABDERHALDEN TESTS EMPLOYING PHASEOLIN (ADZUKI BEAN) AS ANTIGEN

Protein	Number of Tests	"++ Strong" or "++"	"+ Strong" or "+"	Strong Trace	Trace or Faint Trace	Questionable	No Reactions
Phaseolin.....	6	0	3	2	1	0	0
Glycinin.....	6	0	0	2	2	0	2
Legumin (pea).....	6	0	1	2	1	0	2
Legumin (vetch).....	6	0	2	1	3	0	0
Vicilin (pea).....	6	1	2	0	3	0	0
Legumin (lentil).....	6	0	1	0	3	0	2
Viginin (cow pea).....	5	0	1	0	3	0	1

#### G. WITH GLYCININ FROM THE SOY BEAN AS ANTIGEN

*Rabbit 1.*—Injected with a total of 0.7 gm. glycinin from March 15 to April 10, 1916. Blood was drawn and serum obtained April 7, 10, and 13. The sera were all predialyzed to running salt solution (0.85%) for 7, 6 and 7 hours, respectively. The experiment of April 13 was questionable and is discarded for reasons already indicated.

TABLE 26  
RESULTS OF ABDERHALDEN TESTS EMPLOYING GLYCININ (SOY BEAN) AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, I	April 7	22	0.1 glycinin (soy bean).....	1	+
			0.1 phaseolin (adzuki bean).....	1	Trace
			0.1 legumin (pea).....	1	Trace
			0.1 legumin (vetch).....	1	++
			0.1 vicilin (pea).....	1	+
			0.1 legumin (lentil).....	1	+
			0.1 vignin (cow pea).....	1	+
				1*	0
2, II	April 10	19	0.1 glycinin (soy bean).....	1	+
			0.1 phaseolin (adzuki bean).....	1	+
			0.1 legumin (pea).....	1	Trace
			0.1 legumin (vetch).....	1	Trace
			0.1 vicilin (pea).....	1	++
			0.1 legumin (lentil).....	1	+
			0.1 vignin (cow pea).....	1	+
				1*	0

\* Control.

*Rabbit 2.*—Injected with a total of 0.7 gm. glycinin from March 15 to April 10, 1916. Blood was drawn and serum obtained April 7, 10, and 13. The sera were all predialyzed to running salt solution (0.85%) for 7, 6, and 7 hours, respectively.

TABLE 27  
RESULTS OF ABDERHALDEN TESTS EMPLOYING GLYCININ (SOY BEAN) AS ANTIGEN

Test	Date	Incuba- tion, hr.	Substrate, gm.	Serum, c.c.	Reaction
1, III	April 7	22	0.1 glycinin (soy bean).....	1	Trace
			0.1 phaseolin (adzuki bean).....	1	Trace
			0.1 legumin (pea).....	1	Trace
			0.1 legumin (vetch).....	1	Trace
			0.1 vicilin (pea).....	1	Trace
			0.1 legumin (lentil).....	1	Trace
			0.1 vignin (cow pea).....	1	+
				1*	Trace ?
2, IV	April 10	19	0.1 glycinin (soy bean) .....	1	+
			0.1 phaseolin (adzuki bean).....	1	+
			0.1 legumin (pea).....	1	Trace
			0.1 legumin (vetch).....	1	0
			0.1 vicilin (pea).....	1	Trace
			0.1 legumin (lentil).....	1	+
			0.1 vignin (cow pea).....	1	+
				1*	0
3, V	April 13	19	0.1 glycinin (soy bean).....	1	+
			0.1 phaseolin (adzuki bean).....	1	+ Strong
			0.1 legumin (pea).....	1	Strong trace
			0.1 legumin (vetch).....	1	Trace
			0.1 vicilin (pea).....	1	Trace
			0.1 legumin (lentil).....	1	Trace
			0.1 vignin (cow pea).....	1	+
				1*	0

\* Control.

A summary of the experiments with glycinin is given in Table 28.

TABLE 28

SUMMARY OF RESULTS OF ABDERHALDEN TESTS EMPLOYING GLYCININ (SOY BEAN) AS ANTIGEN

Protein	Number of Tests	"++ Strong" or "++"	"+" Strong" or "+"	Strong Trace	Trace or Faint Trace	Questionable	No Reaction
Glycinin (soy bean).....	5	0	4	0	1	0	0
Phaseolin (adzuki bean).....	5	0	3	0	2	0	0
Legumin (pea).....	5	0	0	1	4	0	0
Legumin (vetch).....	5	1	0	0	3	0	1
Vicilin (pea).....	5	1	1	0	3	0	0
Legumin (lentil).....	5	0	3	0	2	0	0
Vignin (cow pea).....	5	0	5	0	0	0	0

#### H. WITH EGG WHITE AS ANTIGEN

In these experiments rabbits were injected intravenously with a 33 $\frac{1}{3}$ % solution of fresh filtered egg white in an 0.85% NaCl solution. The amounts introduced varied from 1.5 c.c. to 10 c.c., and the injections were often repeated as frequently as each day. The experiments were in a sense to serve as controls on those in which vegetable proteins were employed, not because of any close relationship between egg white and these proteins, but to determine whether some interaction might take place between proteins so highly dissimilar. It will have been noted already that the sera of some of the animals immunized to the vegetable proteins seemed to interact with egg white.

*Rabbit 1.*—Injected with a total of 47 c.c. of a 33 $\frac{1}{3}$ % solution of egg white Nov. 5 to 29, 1915. Blood was drawn and serum obtained Nov. 11, 17, 23, and 29. That obtained Nov. 11 gave no reaction with any of the substances employed.

TABLE 29

RESULTS OF ABDERHALDEN TEST EMPLOYING EGG WHITE AS ANTIGEN

Test	Date	Incubation, hr.	Substrate	Serum, c.c.	Reaction
1, I	Nov. 17	20	0.5 gm. coagulated egg white.....	1	++
			1 c.c. egg white (conc.).....	1	++
				1*	Trace
2, II	Nov. 23	19	1 c.c. 5% egg white.....	1	0
			1 c.c. egg white (conc.).....	1	+
			0.61 gm. globulin (squash seed).....	1	0
				1*	Trace
3, III	Nov. 29	19	1 c.c. egg white (conc.).....	1	++
			0.1 gm. legumin (pea).....	1	+
			0.1 gm. gliadin (wheat).....	1	++
			0.1 gm. edestin.....	1	0
				1*	0

\* Control.

Rabbit 2.—Injected with a total of 24 c.c. of a 33 $\frac{1}{3}$ % solution of egg white from Jan. 13 to Feb. 4, 1916. Blood was drawn and serum obtained Feb. 7.

TABLE 30  
RESULTS OF ABDERHALDEN TEST EMPLOYING EGG WHITE AS ANTIGEN

Test	Date	Incubation, hr.	Substrate	Serum, c.c.	Reaction
1, IV	Feb 7	17	1 c.c. egg white (conc.).....	1.7	Strong trace
			1 c.c. egg white (conc.).....	1	Strong trace
			1 c.c. 5% egg white.....	1	0
			1 c.c. 5% egg white.....	1.5	+
			1 c.c. 5% egg white.....	1.5*	0
			0.1 gm. legumin (pea).....	1	+ Strong
			0.1 gm. gliadin (wheat).....	1	0
			0.1 gm. gliadin (rye).....	1	Faint trace
			0.1 gm. edestin.....	1	0
			0.2 gm. edestin.....	1	0
			0.1 gm. globulin (squash seed).....	1	++
			0.1 gm. hordein.....	1	0
			0.1 gm. prolamín (oat).....	1	Faint trace

\* Control.

A summary of the experiments with egg white is given in Table 31.

TABLE 31  
SUMMARY OF RESULTS OF ABDERHALDEN TESTS EMPLOYING EGG WHITE AS ANTIGEN

Protein	Number of Tests	"++ Strong" or "++"	"+ Strong" or "+"	Strong Trace	Trace or Faint Trace	Questionable	No Reaction
Egg white (conc.).....	5	2	1	2	0	0	0
Egg white (5%).....	3	0	1	0	0	0	2
Egg white (coagulated; 0.5 gm.)...	1	1	0	0	0	0	0
Legumin (pea).....	2	0	2	0	0	0	0
Gliadin (wheat).....	2	1	0	0	0	0	1
Gliadin (rye).....	11	0	0	0	1	0	0
Edestin.....	3	0	0	0	0	0	3
Globulin (squash seed).....	2	1	0	0	0	0	1
Hordein.....	1	0	0	0	0	0	1
Prolamin (oat).....	1	0	0	0	1	0	0

GENERAL DISCUSSION

As can be seen from the tables the purified vegetable proteins can be utilized in immunizing animals for the Abderhalden reaction. Sera are thus obtained which when added to various vegetable proteins cause an interaction of some kind, whereby dialyzable products can be detected. The results of these interactions from the individual experiments (Tables 7, 11, 15, 18, 20, 25, 28, and 31) are placed in Table 32 in the form of a general summary.

In analyzing these tables certain facts should be borne in mind. As has been stated, it was not possible to obtain an equal number of reactions for all the proteins employed, altho this would have been highly desirable. Hence it may appear (Table 32) that whereas 16 of 18, or 88% of the tests made with edestin immune serum against edestin were positive, 2 tests, or 100%, using rye gliadin were positive. Obviously conclusions drawn on such a basis would be fallacious. It should be pointed out also that Table 32 includes all the reactions in the various experiments which were read "positive" ranging from "strong trace" to "++ strong." The weaker reactions have been eliminated, for it is here that the greatest uncertainty lies in making the readings.

The performance of a single experiment is beset by many pitfalls, which can be eliminated only after long and diligent labor. The quantitative reading of the color reactions is much governed by subjective influences. We avoided this at times by asking another person to make the readings. Faulty dialyzing tubes no doubt play a serious part as many investigators have emphasized. Indeed, any one who has worked extensively with the dialysis method of Abderhalden is familiar with the laborious care which must be exercised continually and the uncertainty which may attend the most careful efforts. One hesitates to use a method which does not permit of clear cut and definite results.

It is to be noted, however, that the reactions between immune serum and homologous antigen tend to be specific. That is, when a sufficient number of tests have been made to warrant comparison, a higher percentage of the reactions are positive when the specific substrate is employed than when a nonspecific substrate is employed. For example (quoting from Table 32), 16 of 18 tests were positive when edestin immune serum was used against edestin, while 4 of 7 were positive when wheat gliadin was used as substrate. Again, wheat gliadin immune serum reacted 16 times out of 16 with gliadin as substrate, and 3 times out of 11 with edestin as substrate. The individual summaries will indicate that the greater number of the strongest reactions tended to occur with the specific protein, while the greatest number of weaker reactions occurred with other substrates. The totals in Table 32 indicate several interesting features. Thus edestin and hordein and egg white, when tested as substrates against heterologous sera, yield a low percentage of positive results as compared with negative results. Squash-seed globulin, excelsin, gliadin from rye, oat prolamins, pea

TABLE 32

GENERAL SUMMARY OF RESULTS OF ABDERHALDEN TESTS EMPLOYING VEGETABLE PROTEINS AS ANTIGEN

Proteins Employed as Sub- strates	Table References	Substrates																										
		Edestin			Globulin (Squash Seed)			Excelsin			Gliadin (Wheat)			Gliadin (Rye)			Hordein			Pro- lamin (Oat)			Alcohol- Soluble Protein from Millet			Globulin (Oæsto Bean)		
		No. of Tests	Positive	Negative	No. of Tests	Positive	Negative	No. of Tests	Positive	Negative	No. of Tests	Positive	Negative	No. of Tests	Positive	Negative	No. of Tests	Positive	Negative	No. of Tests	Positive	Negative	No. of Tests	Positive	Negative	No. of Tests	Positive	Negative
Edestin.....	7	18	16	1*	6	1	5	7	2	4	7	4	2	2	2	0	7	1	6	2	1	1	1	0	1	1	0	1
Globulin.... (squash seed)	11	9	0	9	15	7	6	10	4	6	9	5	2	5	1	4	7	2	5	2	0	1	.....	.....	.....	1	1	0
Ghadin..... (wheat)	15	11	3	4	10	4	5	.....	.....	.....	16	16	0	9	6	3	10	1	6	8	4	3	1	0	1	.....	.....	.....
Hordein....	18	5	2	2	5	3	1	.....	.....	.....	5	4	1	4	2	2	10	8	2	3	2	1	.....	.....	.....	.....	.....	.....
Legumin.... (pea)	22	8	3	5	8	3	4	.....	.....	.....	8	6	2	.....	.....	.....	7	2	4	2	0	1	.....	.....	.....	1	1	0
Phaseolin...	25	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Glycinin....	28	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Egg white...	31	3	0	3	2	1	1	.....	.....	.....	2	1	1	1	0	0	1	0	1	1	0	0	.....	.....	.....	.....	.....	.....
Totals†...	..	36	8	23	31	12	16	17	6	10	31	20	8	21	11	9	32	6	22	18	7	7	2	0	2	3	2	1

\* Questionable and trace or faint-trace reactions are not recorded.

† The totals do not include the reactions between an immune serum and its homologous substrate; for example, edestin immune serum as tested against edestin.

legumin, glycinin, legumin from vetch and lentil, and vignin have approximately as many positive as negative reactions when used as substrates against heterologous sera. Gliadin from wheat, phaseolin, and vicilin, however, have a large percentage of positive reactions as compared with negative reactions.

The experiments have moreover shown that a rabbit may react in an absolutely specific manner at one time (Experiment V, Table 2), altho it has not done so in a previous test and does not do so in the serum obtained subsequently. The individual rabbits also appear to differ in their reacting powers. Lake,<sup>2</sup> working out the immunologic relationship of certain of the vegetable proteins as shown by complement-fixation, passive anaphylaxis, and precipitin reactions, states that "anti-sera to the same protein obtained from different individual animals differ in their relations, for some unknown cause;" also, "anti-serum at one stage of its development may be apparently of sharply limited specificity, etc." He indicates that increased antibody content in the serum will cause the latter to react more generally. On the other hand, Pearce and Williams,<sup>7</sup> using kidney tissue as antigen, con-

TABLE 32—*Continued*

GENERAL SUMMARY OF RESULTS OF ABDERHALDEN TESTS EMPLOYING VEGETABLE PROTEINS AS ANTIGEN

Substrates										
Cocoa-nut Protein	Globulin (Black Walnut)	Legu-min (Horse Bean)	Legu-min (Pea)	Phaseo-lin	Glyce-lin	Legu-min (Vetch)	Vicilin (Pea)	Legu-min (Lentil)	Vignin (Cow-pea)	Egg White
No. of Tests Positive Negative	No. of Tests Positive Negative	No. of Tests Positive Negative	No. of Tests Positive Negative	No. of Tests Positive Negative	No. of Tests Positive Negative	No. of Tests Positive Negative	No. of Tests Positive Negative	No. of Tests Positive Negative	No. of Tests Positive Negative	No. of Tests Positive Negative
1 1 0 1 0 1	1 0 1 .....	1 0 1 .....	7 2 4 9 4 3	..... .....	..... .....	..... .....	..... .....	..... .....	..... .....	6 0 6 3 0 2
.....	.....	.....	10 5 3	.....	.....	.....	.....	.....	.....	5 0 3
.....	.....	.....	5 1 2	.....	.....	.....	.....	.....	.....	3 2 1
.....	.....	6 4 1	18 17 0	5 1 1	8 1 3	8 2 3	8 5 1	6 2 2	4 2 1	3 0 2
.....	.....	.....	6 3 2	6 5 0	6 2 2	6 3 0	6 3 0	6 1 2	5 1 1	
.....	.....	.....	5 1 0	5 3 0	5 4 0	5 1 1	5 2 0	5 3 0	5 5 0	
.....	.....	.....	2 2 0	.....	.....	.....	.....	.....	.....	9 7 2
2 1 1	1 0 1	7 4 2	44 18 14	10 4 1	14 3 5	19 6 4	19 10 1	17 6 4	14 3 2	20 2 16

clude that "multiple injections tend to a slight selective action." Our results at least indicate that continued injection did not increase the specificity of the serum.

The results obtained by means of the Abderhalden reaction have been so irregular and uncertain that a detailed comparison of the results obtained by this means with the observations on the same proteins made in respect to anaphylaxis and other reactions by Wells, Osborne, and Lake,<sup>2</sup> seems scarcely profitable at this time. I have made a careful comparison of the data published by these observers with those reported in this paper, and have found that while in many instances there is a reasonable agreement, in others there are great and unaccountable discrepancies.

## SUMMARY OF EXPERIMENTS

The action of immune sera on purified vegetable proteins as determined by the Abderhalden reaction seems to be only quantitatively specific. Immune sera tend to react more often and more strongly with their specific substrates than with any other substrate. However, the reaction is far from being absolutely specific.

An immune serum may or may not react specifically with its own antigen; in the same test it may not react at all with its antigen, while reacting with other proteins.

Immune sera against closely allied proteins tend to interact with these various antigens. This is especially demonstrated by the inter-reactions among the proteins from the leguminous seeds.

A greater degree of specificity exists between animal proteins (egg white) and vegetable proteins than between different vegetable proteins.

The serum of an immune rabbit is not constant in its reactive power over a continued period, nor in the degree of specificity it exhibits. There also exists an apparent difference between the sera of the individual rabbits in these regards.

#### GENERAL CONCLUSIONS

It is demonstrated in this work that the specificity of the Abderhalden reaction (dialysis method) in experimental animals (rabbits) immunized with pure isolated vegetable proteins is far from being absolute. The conditions under which this experimental work was performed were rigidly controlled and the requisite care was constantly exercised in performing all the Abderhalden tests. The biologic interrelationship and specificity of the preparations of vegetable proteins used for this work have been previously tested by means of anaphylaxis, complement-fixation, and precipitin reactions.

Under the conditions of the experiments the Abderhalden reaction is at best only quantitatively specific and even this quantitative specificity is not always exhibited. This is demonstrated by the following observations: (1) an homologous substrate may react specifically with its immune serum, no other protein reacting; (2) it may vary quantitatively in the degree of interreaction with its own immune serum; (3) it may react with its immune serum but no more strongly than do the heterologous substrates tried against the same immune serum; (4) it may give no reaction against its immune serum, while other heterologous proteins may react strongly against this serum; (5) it may react at times more strongly with a heterologous immune serum than with its own immune serum. However, there is an obvious tendency for a substrate to react more often and yield stronger reactions when tested against its homologous immune serum, than when tested against a heterologous immune serum.

When the test is made with closely allied pure vegetable proteins, the Abderhalden reaction tends to be less quantitatively specific the more similar these same proteins are. However, there is no absolute specificity even between widely diverse proteins, such as egg white and pure vegetable protein, as tested in our experiments.

In comparing the results obtained by the Abderhalden test with those obtained by anaphylaxis, by Wells, Osborne and Lake, using the same pure vegetable proteins in each case, it was found that the results present certain resemblances, but also often definite differences. In either reaction the more closely the proteins are allied chemically and physically, the less specific the reactions tend to become. On the whole, however, the results obtained by anaphylaxis are much more constant and specific.

## THE COMPLEMENT CONTENT OF ECK-FISTULA DOGS \*

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Many investigators have attempted to associate the liver with the production of complement.

Ehrlich and Morgenroth<sup>1</sup> observed a diminution of complement in dogs subjected to phosphorus poisoning with subsequent degeneration of the liver. Extirpation of the liver has been attempted by Nolf<sup>2</sup> and others, but in view of the almost insurmountable difficulties of the operation the work has been wholly unsatisfactory. Nolf next attempted to establish Eck's fistula in rabbits. Anastomosing the vena cava with the portal vein he obtained an immediate drop in complement, which lasted until the death of the animals 3 or 4 hours after the operation, and he concluded from this that the liver played an important rôle in the production of the complement. His work was later confirmed by Müller,<sup>3</sup> who also perfused serum through the liver and observed an increase in complement. Liefmann<sup>4</sup> was unable to confirm the results obtained by Müller. As a further check on the work he extirpated the liver from frogs, but observed no diminution of complement, the animals living, in some cases, 1 week.

More recently Dick<sup>5</sup> produced necrosis of the liver in dogs by means of chloroform poisoning, as suggested by Richards and Howland.<sup>6</sup> He observed a progressive drop in complement, lasting until the death of the animals some 48 hours afterwards. The complement content of normal dog serum was observed to vary from one-third to one-twelfth that of guinea-pig serum. The complement content of individual dogs showed a similar variation when the serum was titrated from time to time. In the animals suffering from chloroform poisoning, the complement content dropped to one-fortieth that of guinea-pig serum in 46 hours, just before the death of the animals.

Dick also produced destruction of liver cells by means of hydrazin sulfate. At the beginning of the experiment the complement content was one-fifth, after 48 hours it was one-tenth, while after 72 hours it was one-fortieth that of guinea-pig serum. The dog died a few hours later. Extensive necrosis of the liver was found microscopically. Dick concludes that complement is a proteolytic ferment which is either formed in the liver or is dependent on liver activity for its presence in the blood.

\* Received for publication June 30, 1916.

<sup>1</sup> Gesammelte arbeiten zur Immunitätsforschung, 1904.

<sup>2</sup> Bull. de l'Acad. Roy. de Belg., 1908, Classe des Sc., p. 748. For a more complete bibliography of the work done on complement, see Zinsser's Infection and Resistance, 1914, and also the article by Dick.<sup>5</sup>

<sup>3</sup> Centralbl. f. Bakteriöl., I, O., 1911, 57, p. 577.

<sup>4</sup> Weicharts Jahresbericht, 1912.

<sup>5</sup> Jour. Infect. Dis., 1913, 12, p. 111.

<sup>6</sup> Jour. Exper. Med., 1909, 11, p. 344.

It will be observed that in all the experiments cited, the animals died within a few hours or at most a few days after the work was started. In the work of Nolf and others on Eck's fistula only 3 or 4 hours elapsed before the animals succumbed, while Dick's chloroformed animals lived only about 48 hours.

In view of the fact that dogs in which Eck's fistula had been established by Dr. S. A. Matthews and one of us of the department of physiology, were surviving in good health almost indefinitely, it was thought worth while to study the complement content of these animals and to compare it with the progressive and noticeable atrophy, fatty degeneration and infiltration, and necrosis of the liver which followed the operation. For this work those dogs were selected which had no normal antihuman or antisheep hemolysins in their sera.

Blood was obtained from the external saphenous vein from one to several times preceding the operation and the complement content determined and compared with that of guinea-pig serum. Two hundredths cubic centimeter, that is, 0.2 c.c. of a 1:10 dilution, of guinea-pig complement was adopted as a unit for comparison.

The estimation of complement was carried out as follows:

At first both antihuman and antisheep hemolytic amboceptors from rabbits immunized against the respective cells were employed. These were titrated to determine the unit of each. In the case of the antihuman hemolytic amboceptor, we used the method of titration recommended by Noguchi<sup>7</sup> and others. Each hemolytic test tube contained 0.1 c.c. of a 10% suspension of washed human red-blood corpuscles, 0.1 c.c. of its respective dilution of amboceptor, 0.5 c.c. of a 1:10 dilution of active guinea-pig complement, and enough physiologic salt solution to make the final volume 1 c.c. These tubes were incubated in a water bath at 37.5 C. for 30 minutes. It was found that the tube containing 0.1 c.c. of 1:120 dilution of amboceptor held the highest dilution showing complete hemolysis. This was considered the titer of the serum. In titrating dog complement, each hemolytic tube contained 0.1 c.c. of a 10% suspension of washed human red-blood corpuscles, 1 unit of amboceptor, its respective amount of dog serum, and physiologic salt solution to make the volume 1 c.c. These tubes were then incubated in a water bath at 37.5 C. for 30 minutes and examined. They were next placed in the ice box over night and in the morning read again. The least amount of dog serum bringing about complete hemolysis was considered the titer of the dog complement.

In titrating dog serum varying amounts of a 1:10 dilution, as well as varying amounts of straight serum, were used. Inasmuch as 0.02 c.c. of guinea-pig complement was the unit for comparison, if it was found that 0.1 c.c. of straight dog serum was the titer of the dog complement, then the latter would be one-fifth as strong as guinea-pig complement.

In working with antisheep amboceptor, 0.5 c.c. of a 1:1000 dilution was found to be a unit; that is, it produced complete hemolysis in a tube containing 0.5 c.c. of a 5% suspension of washed sheep corpuscles, an excess of complement,

<sup>7</sup> Serum Diagnosis of Syphilis and the Butyric Acid Test for Syphilis, 1910.

and sufficient salt solution to make the final volume 2.5 c.c. Complement was then titrated against this unit of amboceptor.

In most of the work the antihuman hemolytic amboceptor only was used, as it was very satisfactory.

The titer of the dog complement was then determined for several days preceding and immediately before and after the operation. It was then titrated 2½, 6, 16, and 24 hours after the operation and at frequent intervals thereafter until the dogs were killed and the liver removed for microscopical examination. Animals were killed and tissue was obtained for study 30, 60, and 90 days after Eck's fistula had been established. Liver tissue from apparently normal dogs was obtained for comparison.

Table 1 records the results of the titrations. The data on Dogs 7 and 8 are also shown in the form of curves in the chart.

TABLE 1  
THE COMPLEMENT TITER IN ECK FISTULA DOGS

Dog	Titer of Dog Complement in Terms of Guinea-Pig Complement									Anesthetic Used	Remarks
	Immediately Before Operation	Immediately After Operation	2½ Hours After Operation	6 Hours After Operation	16 Hours After Operation	24 Hours After Operation	30 Days After Operation	60 Days After Operation	90 Days After Operation		
1	1/8	....	....	....	....	1/5	1/5	....	....	Ether	Posted 30 days
2	1/5	....	....	....	....	1/5	1/8	....	....	Ether	Posted 30 days
3	1/10	....	....	....	....	1/12	....	....	....	Ether	Killed by mistake
4	1/5	1/10	1/10	1/8	1/5	1/5	1/5	1/5	....	Ether	Posted 60 days
5	1/10	1/15	1/12	1/10	1/10	1/8	1/5	1/8	1/5	Ether	Posted 90 days
6	1/6	1/12	1/10	1/8	1/5	1/5	1/5	1/8	....	Ether	Still living
7	1/5	1/10	1/8	1/8	1/5	1/5	1/5	1/8	....	Ether	Still living
8	1/8	1/30	1/15	1/5	1/8	1/5	1/5	....	....	Chloroform	Died from ammonia poisoning
9	1/4	1/25	1/15	1/5	1/5	1/5	1/5	1/10	....	Chloroform	Still living

The titer of the complement of individual normal dogs has been observed to fluctuate from one-fourth to one-twelfth that of guinea-pig complement.

Microscopically the livers of the dogs examined at 30, 60, and 90 days after the establishment of Eck's fistula, disclosed a progressive fatty degeneration and infiltration with necrosis of the liver cells. There was no corresponding decrease in complement accompanying the degenerative processes in the liver. Whether there will occur a drop in complement when the liver has completely degenerated, remains to be determined. Dogs are being carried over until next year for that purpose.

Ether was used as an anesthetic in 7 of the operations. In the case of the first 3 dogs, data on the complement unfortunately were not obtained until 24 hours after the operation when it was back to normal. In the next 4 dogs, immediately following the operation there was a drop in complement of about one-half, the complement returning to normal within 16 hours. In the 2 dogs operated upon under chloroform anesthesia the drop was very much greater, the titer going down to approximately one-fourth to one-sixth of the titer before the operation and returning to normal within 6 hours after the operation.

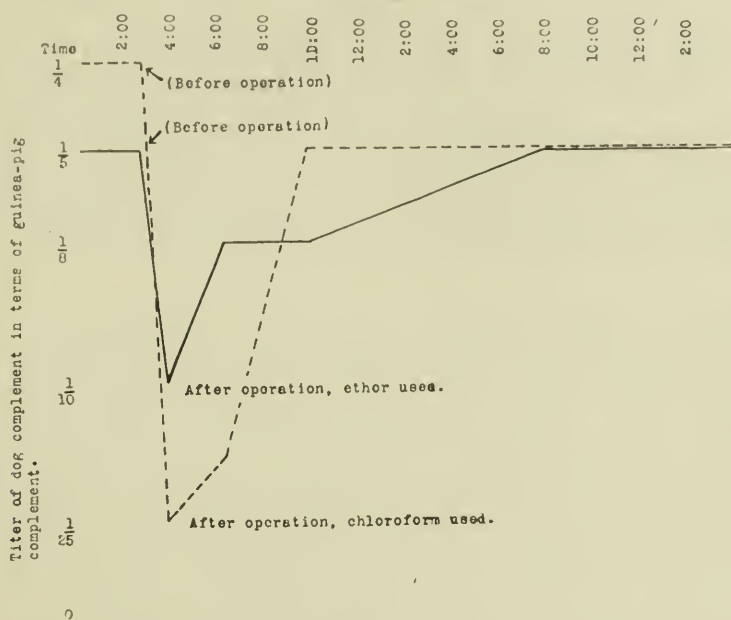


Chart 1. The broken line shows the titer of complement before and after the establishment of Eck's fistula when chloroform was used as the anesthetic. The solid line shows the titer of complement before and after the establishment of Eck's fistula when ether was used as the anesthetic.

We have here several factors all or part of which may contribute to the temporary drop in complement: (1) the interference with the normal function of the liver, (2) the sudden shunting of the portal circulation into the general circulation, (3) the effect of the anesthetic on the various tissue cells, leading not only to disturbed function but to change of blood constituents, (4) the presence of the anesthetic in the circulating blood in contact with the complement, and (5) the shock of the surgical operation. In view of the fact that the drop in complement was much less under ether than it was under chloroform anesthesia, it would appear that the 3rd and 4th factors may be largely responsible for the immediate fall in complement.

In their work on Eck-fistula dogs, Matthews and Miller<sup>8</sup> emphasized the danger of the formation of adhesions following the operation, and the establishment of a collateral circulation for the liver. In the work here recorded great care was exercised to avoid this possibility. Adhesions were looked for carefully post mortem and data on dogs showing adhesions and collateral circulation were not included in the report. A few weeks after the operations the feces of the dogs became grayish-white in color, indicating the absence of bile. Symptoms of ammonia poisoning, as pointed out by Hahn, Massen, Nenchi, and Pawlow,<sup>9</sup> and Matthews and Miller,<sup>8</sup> could be easily induced by increasing the amount of meat in the diet. These were fair indications that a collateral circulation had not been established. In a few cases hemolytic substances appeared in the blood, but these could be eliminated easily by putting the dogs on a bread and water or milk diet for a couple of days. The complement was usually at a low normal, that is, one-eighth to one-twelfth that of guinea-pig serum, but normal dogs showed a corresponding drop in complement when put on the same diet.

In regard to tissue changes in the liver following the Eck-fistula operation, Matthews and Miller make the following statement: "It is well known that after an Eck's fistula the liver soon begins to undergo fatty necrosis, which eventually invades the whole organ. This change in the nutrition of the organ may be responsible, in large measure, for the changes in metabolism so noticeable after an Eck's fistula." Macroscopically the liver showed a progressive atrophy. The rate of degeneration varied with different dogs, but was usually almost at a maximum in about 90 days. However, Dog 5 had not reached that stage at 90 days. In Dog 9, which at the writing of this report had been carried along 70 days, the liver was not palpable. The complement content on the 70th day was one-tenth that of guinea-pig serum. This dog, as well as normal dogs, had been kept off meat and fed on table scraps for 4 days previous to the titration. The sera of the normal dogs showed a complement content of one-tenth to one-twelfth that of guinea-pig serum at this time; previous to the change of diet it had been one-fifth.

#### CONCLUSIONS

The complement content of normal dog serum may vary in the same animal from one-fourth to one-twelfth that of guinea-pig serum.

<sup>9</sup> Arch. f. Exper. Path. u. Pharm. 1893, 32, p. 161.

<sup>8</sup> Jour. Biol. Chem., 1913, 15, p. 87.

Immediately following the establishment of Eck's fistula there is a temporary drop in complement lasting not more than 16 hours as a rule.

There is a much greater drop in complement under chloroform anesthesia than under ether anesthesia. The return to normal is apparently just as rapid when only sufficient chloroform is used to produce the surgical anesthesia.

The complement content of dog serum following the establishment of Eck's fistula was normal within from 6 to 16 hours after the operation and remained normal for at least 90 days (last observation) in the dogs studied.

There is no corresponding drop in complement paralleling or accompanying the degeneration of the liver.

There does not seem to be sufficient evidence, as yet, to warrant the assumption that the liver plays a more important rôle in the production of complement than do other organs or tissues of the body.

# THE PERMEABILITY OF THE GASTRO-INTESTINAL WALL TO INFECTION WITH SPOROTHRIX SCHENCKII \*

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The permeability of the mucosa of the gastro-intestinal tract to organisms of various kinds may be considered a phase of the general problem of focal infections.

Adami<sup>1</sup> years ago emphasized the constant passage of bacteria through apparently uninjured mucosa into the lymphatics and the blood stream. He showed that bacteria are found normally in various stages of disintegration especially in the lymph glands, spleen, and liver. He thought many of these organisms which appeared as diplococcoid forms were probably *B. coli*. Adami so well summarized his views based on his observations and on the reports in the literature at that time that I quote as follows:

"1. Normally there is a passage out of leukocytes through the mucosa on to the free surface of, more especially, the alimentary tract.

2. These leukocytes, while in part undergoing a destruction, in part find their way back between the epithelial cells, bearing with them foodstuffs and solid particles, among which may be the bacteria present in the cavity of the gut.

3. During the active congestion which accompanies digestion, the passage out and return of these wandering cells is increased.

4. These cells upon their return find their way either into the lymphatic channels or the venules of the portal system.

5. In either position, they tend to be destroyed and digested by the leukocytes and thus, while preparations of the mesentery and of the mesenteric lymphatic glands may show abundant bacteria, the vast majority of these at the same time show obvious degeneration, while cultures made from the mesentery or from the lymphatic glands upon ordinary media by ordinary methods as a consequence tend to remain sterile. Similarly in the normal liver, the same rapid destruction takes place, so that here again, by ordinary methods, no evidence of living bacteria is obtainable.

6. By the employment of adequate methods it can be demonstrated that even in the healthy liver and kidney in a large number of cases, in one animal at least—the rabbit—a certain number of living microbes are present at any one moment so that, if the healthy organ be removed from the body, cultures can be obtained of these living microbes.

7. It is most probable, that in ordinary health a certain number of bacteria which have not been destroyed by the leukocytes or removed by the lymphatic glands or endothelium of the portal system, pass either through the thoracic

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<sup>1</sup> Jour. Am. Med. Assn., 1899, 33, p. 1509.

duct or through the liver into the systemic blood. Such bacteria tend to be removed more especially by the kidneys, though it may be by other glandular organs. In any case the ordinary methods at present employed in making cultures from the blood are inadequate to detect the presence of such bacteria unless they are of such a nature or are circulating in such quantities that the whole number is not destroyed by the bactericidal power of the shed blood."

Adami here also discusses latent infections and the condition he calls sub-infection, his views having a direct bearing on the general problem of focal infection as developed in more recent literature.

Indicative of the protective processes going on in the intestinal canal against invading organisms is the occurrence of certain types of inflammatory cells in the mucous membrane. At birth the pharyngeal and intestinal mucosa is sterile, but within the first 24-48 hours the canal becomes infected. For the first few days there are no inflammatory cells to speak of in the mucosa. Various types of such cells, especially plasma cells, then slowly appear in the mucosa and are always found thereafter. Such cells are especially numerous in certain parts of the alimentary canal, namely, the tonsils<sup>2</sup> and the appendix. No doubt one of the functions of such cells is that of protection against the infected intestinal contents, from which in accordance with the views of Adami and others bacteria are being absorbed.

In keeping with these observations is the fact that in new-born animals the permeability of the intestine is greater than in older animals. Von Behring<sup>3</sup> showed that ingested attenuated anthrax bacilli would infect new-born guinea-pigs, and Plate<sup>4</sup> reported that guinea-pigs 5 days old, when fed tubercle bacilli, developed tuberculosis whereas older guinea-pigs remained uninfected.

That bacteria pass through the apparently uninjured gastro-intestinal mucosa is a well-recognized fact experimentally. Ravenel infected guinea-pigs with chyle from dogs which a few hours previously had ingested tubercle bacilli with a large amount of fat.<sup>5</sup> Many workers have demonstrated that tubercle bacilli may pass through or between the epithelial cells and thence into the lymphatic glands and thoracic ducts without leaving any trace at the point of entry. It is true, however, that in order to obtain such experimental infections with tubercle bacilli enormous quantities must be ingested.

In this paper I wish to present certain experimental data bearing on the subject of the permeability of the alimentary mucosa to the

<sup>2</sup> Davis: *Jour. Infect. Dis.*, 1912, 10, p. 142.

<sup>3</sup> *Deutsch. med. Wehnschr.*, 1903, 29, p. 689.

<sup>4</sup> *Inaugural Dissertation*, 1905.

<sup>5</sup> *Jour. Med. Research*, 1904, 10, p. 460.

pathogenic organism *Sporothrix schenckii*. The experiments were designed, first, to test the permeability of the intestinal mucosa to a nonmotile organism many times larger than the bacteria which have been chiefly used heretofore in such experiments; second, to determine whether or not it is possible for sporotrichosis to be transmitted in this way from one animal or person to another.

The organism used was a typical *Sporothrix schenckii* isolated originally from a man living in North Dakota. The details of this case including a description of the organism have been reported by Hyde and Davis.<sup>6</sup>

The experiments were all made with the white rat because of its high susceptibility to infection with this organism when inoculated subcutaneously or intraperitoneally, and also because of its readiness to feed upon various preparations and media containing large numbers of these organisms. Young animals about three-fourths grown were used. They were kept in small compartment cages, always but one animal to a compartment to avoid, as far as possible, infection through bites and scratches.

As a routine the organisms were grown on agar-agar for about 10 days or 2 weeks. The growth, which as a rule is rather tough and leathery, was removed from the surface of the media, placed in a small mortar, and ground up. Later it was thoroughly mixed with from about 5 to 10 c.c. of milk. Mixing with other food was tried but milk was found to be the best vehicle. The rats readily consumed the organisms when thus prepared, especially if they had not been fed during the previous 12 hours.

Tests were made to determine the length of time the sporotricha remained alive in the stomach of the rat. At the end of 2 hours after the ingestion of the milk suspension, the spores and mycelium were always to be seen in the smears of the curdled milk, and by culture on suitable sugar media the organisms were readily recovered. At the end of 3 hours they could usually be recovered from the stomach. In the case of 4 rats, the stomach contents of which I examined at the end of 4 hours, I could not detect the organisms in smear or in culture. It should be pointed out that these observations were made on rats the stomachs of which were practically empty when the milk suspension was ingested.

A number of animals died within a short period of time following the feeding. Two different sets of 6 rats each died within from 24 to 48 hours after eating large quantities of cultures of the sporothrix. Occasionally in other sets of animals an individual would die. The stomach wall was in some instances red and the intestinal mucosa hyperemic. Examination of the heart blood and other body fluids did not reveal any sporothrix organisms. The young rats were more

<sup>6</sup> Jour. Cutan. Dis., 1910, 28, p. 321.

susceptible in this regard than the older animals. I suspect that they died from toxic substances derived from the organism or free in the cultures, but I am not sure that this is the true explanation of these rapid deaths. I am satisfied that they were not real sporothrix infections.

After eliminating a number of experiments which were unsatisfactory, I have observations on 13 animals which were fed cultures regularly over a period of several weeks or months. The data are summarized in Table 1. The amount of culture consumed by a rat in each feeding varied somewhat but was usually the growth from the surface of 1 agar slant, in some of the feedings one-half of this amount. It may be stated that this amount of growth when mixed in milk as described makes a fairly heavy suspension, a smear from which reveals in a single field hundreds of spores and many filaments.

TABLE 1  
RESULTS OF FEEDING WHITE RATS WITH SPOROTHRIX SCHENCKII

Animal	Dates of Feeding	Number of Feedings	Period of Feeding	Date of Autopsy	Results
1	Apr. 29, May 12, 29.....	3	4 weeks	June 10	Negative
2	Apr. 29, May 12, 29.....	3	4 weeks	June 10	Negative
3	Apr. 29, May 12, 29.....	3	4 weeks	June 7	Positive
4	Apr. 29, May 12, 29.....	3	4 weeks	June 7	Positive
5	Sept. 26, Oct. 7, 20, Nov. 2, 4, 27, Dec. 8, 10, Jan. 20, 28.....	10	16 weeks	Feb. 24	Negative
6	Sept. 26, Oct. 7, 20, Nov. 2, 4, 27, Dec. 8, 10, Jan. 20, 28.....	10	16 weeks	Mar. 15	Negative
7	Sept. 26, Oct. 7, 20, Nov. 2, 4, 27, Dec. 8, 10, Jan. 20, 28.....	10	16 weeks	Mar. 23	Negative
8	Nov. 2, 3, 7.....	3	5 days	Nov. 16	Negative
9	Nov. 2, 3, 7, 27, Dec. 8, 10, Jan. 20, 28.....	8	11 weeks	Mar. 16	Negative
10	Nov. 2, 3, 7, 27, Dec. 8, 10, Jan. 20, 28.....	8	11 weeks	Feb. 24	Negative
11	Nov. 7, 27, Dec. 8, 10, Jan. 20, 28.....	6	10 weeks	Mar. 16	Negative
12	Nov. 7, 27, Dec. 8, 10, Jan. 20, 28.....	6	10 weeks	Mar. 18	Negative
13	Nov. 7, 27, Dec. 8, 10, Jan. 20.....	5	10 weeks	Feb. 16	Negative

Rats 3 and 4 on autopsy revealed definite and distinct lesions; these consisted of characteristic nodules in the omentum, the mesentery, and the lymph glands. The liver was not involved, tho lymph glands adjacent to it were. The spleen in one contained several nodes. Smears from these showed the typical elongated tissue forms, and cultures gave pure growths of the sporothrix. Infection of the lungs or thorax did not occur, nor any lesions of the skin.

A careful examination of the gastro-intestinal tract was made in every rat. In no instance were alterations, either active or healed, found in the gastro-intestinal mucosa or wall. It would appear, therefore, that at times the organisms pass through the intestinal mucosa

without causing any appreciable local lesion, and localize in the neighboring lymph glands and other adjacent structures. In order to obtain further evidence on this point, I fed 6 very hungry rats with large quantities of the organisms in milk and at the end of from 2 to 4 hours killed the animals and made cultures of the heart blood and of the mesenteric and neighboring lymph glands. From this set of animals, however, all the culture tubes remained sterile indefinitely.

The organisms fed in these experiments were highly virulent for the rats. This had been determined a short time previously by inoculating a few drops of the suspension of the organisms into the peritoneal cavity or subcutaneously. Invariably infection occurred, terminating in a general and fatal infection in the course of a few weeks.

It is to be noted that the ingestion of large quantities of culture of the sporothrix is necessary in order to produce infection. As before stated, this is likewise true of the tubercle bacillus. When one considers the relative sizes of the two organisms it would seem reasonable to believe that the large spores of the sporothrix would pass through an intact epithelium with even far greater difficulty than would tubercle bacilli.

In this connection it may be mentioned that sporotricha are readily taken up by leukocytes both in vitro and in vivo. It is possible that the organisms were carried through the wall of the intestine by the migration of phagocytes. The recent work of Rous and Jones<sup>7</sup> showing the protective action of cells on intracellular organisms has a direct bearing on this point.

It has been shown by a number of observers that bacteria suspended in fat more readily pass through the mucosa into the chyle vessels than when fat is not present. It is possible that in the experiments here reported the fat in the milk played some rôle in the passage of the organisms through the mucous membrane of the rats. It is not contended however that this is the mechanism by which bacteria usually penetrate the intestinal wall. It may simply facilitate the process. M. Neisser<sup>8</sup> denies that bacteria penetrate the intestinal mucosa, his experiments on this point having proved completely negative.

Apparently no other experiments are recorded on this question of alimentary infection with *Sporothrix schenckii*. Gougerot<sup>9</sup> mentions having fed to rats and young guinea-pigs cultures of *Sporo-*

<sup>7</sup> Jour. Exper. Med., 1916, 23, p. 601.

<sup>8</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1896, 22, p. 12.

<sup>9</sup> Bull. et mém. soc. méd. d. Hôp. de Paris, 1909, 27, p. 909.

thrix *beurmanii*. Two rats were fed with large amounts of such cultures for 6 months. Both animals became infected, and after 10 months one died and the other was killed. Extensive sporotrichotic lesions were found in both. Gougerot cites cases of pharyngeal sporotrichosis which he considers examples of infection with *Sporothrix beurmanii* through the mucous membrane. He mentions finding sporothrix organisms on the salad that the patient was eating, but is unable to say that the infection originated from this source.

As regards the bearing of clinical data on this subject, it is of course true that most cases of infection with *Sporothrix schenckii* result from injuries to the cutaneous surface. I do not know of any of the many cases of this infection now reported in this country in which the evidence indicates definitely that the atrium of infection was the gastro-intestinal tract. In cases of obscure origin this possibly should be considered.

#### SUMMARY

White rats fed at intervals of a few days with large quantities of cultures of *Sporothrix schenckii* may become infected.

The infection tends to localize in the mesentery, peritoneum, and spleen.

The organisms appear to penetrate the normal mucosa of the intestinal tract. No lesions active or healed were visible in the mucosa or in the wall of the stomach or intestines.

# THE RELATION OF ALLANTOIN EXCRETION TO LEUKOPENIA AND LEUKOCYTOSIS IN RABBITS \*

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It has been known for many years<sup>1</sup> that the intravenous injection of the products of protein degeneration or of certain bacteria or their autolysates causes a very prompt and marked leukopenia in the peripheral circulation, followed in a few hours by a considerable leukocytosis. Later studies<sup>2</sup> have led to the conclusion that the leukopenia is the result of the accumulation of great numbers of polymorphonuclear leukocytes in the internal circulation, especially in the liver, spleen, and lungs. There has been, however, no explanation of the purpose of this localization of leukocytes except that of Bull,<sup>3</sup> who ascribes the removal of living typhoid organisms introduced into the circulation to phagocytosis by the polymorphonuclear cells accumulated in the central organs. This explanation is not entirely satisfactory. Nor is it known whether these cells eventually re-enter the general blood stream to take part in the leukocytosis which follows the leukopenia or whether they are all or in part destroyed in the internal circulation. There is some little evidence that tends to the belief that at least some of the leukocytes are destroyed. For example, in several of our experiments examination of the internal organs at the low point of the leukopenia failed to reveal either in smears or sections the usual accumulation of leukocytes. Again the Arneth count of the polymorphonuclear leukocytes made during the period of leukocytosis shows so great an increase in the percentage of young forms as to suggest that at least a part of the matured forms had not returned to the circulation. It must be remembered, however, that both the leukopenia and the leukocytosis are the result very largely of changes in the number of polymorphonuclear leukocytes in the peripheral blood.

\* Received for publication July 18, 1916.

<sup>1</sup> Löwit: Studien zur Physiologie und Pathologie des Blutes und der Lymph, 1892.

<sup>2</sup> Goldscheider and Jacob: Ztschr. f. klin. Med., 1894, 25, p. 373.

<sup>3</sup> Jour. Exper. Med., 1915, 22, p. 475.

This question of the fate of the leukocytes is of importance at the present time on account of its bearing on the recently advanced treatment of various infections by injections of nonspecific vaccines or even of pure proteoses. It seemed to us possible in view of the magnitude of the changes in the leukocyte count that quantitative determination of the excretion of the end products of nucleic-acid metabolism might throw some light on this problem or at least show variations which could be correlated with the changes in the leukocyte count.

The relation of the end products of nucleic-acid metabolism to leukocytosis and to leukocytic destruction is still unsettled. Horbaczewski first advanced the theory that ingested substances increase the excretion of uric acid only in proportion to the leukocytosis they excite, and he believed that dead leukocytes were the source of the uric acid. On the other hand, Plimmer, Dick and Lieb<sup>4</sup> observed that the increase in the excretion of the uric acid after the ingestion of substances causing leukocytosis is present only during the increase of leukocytes in the blood, and they advanced the theory that the uric acid is a product of the metabolism of living leukocytes. Certainly some definite relation does exist and it has often been shown that an increased excretion of uric acid occurs after the ingestion of nucleic acid and in leukemia, but it must be remembered that a similar increase occurs in fever and in many other pathologic disturbances.

#### METHODS

Rabbits were used and these were kept in metabolic cages on a constant daily diet of 200 gm. of carrots, which they ate completely even on the days of treatment. Catheterization was carried out daily just before feeding, except when noted otherwise in the tables. Injections were given in the marginal ear vein and blood counts were taken from the veins of the other ear. Typhoid bacilli were used for injection on account of the uniformity of leukocytic reaction which has been shown to follow intravenous introduction. Cultures from a stock strain of *Bacillus typhosus* were made on agar slants the day before injection and grown at 30 C. for 24 hours. The growth was washed off in salt solution, and if killed, was heated at 60 C. for 1 hour. The number injected was merely estimated roughly as from one-tenth to one-half of a slant. The blood counts were made according to the usual technic. All injections were given about 4 hours after feeding.

The 24-hour urine was collected under toluol. Total nitrogen was determined by the Gunning-Kjeldahl method and urea by the urease method and by Benedict's method. According to the suggestion of Plimmer and Skelton,<sup>5</sup> the difference between the figures obtained by these two methods was accepted as representing the greater part of the allantoin nitrogen. It has been found

<sup>4</sup> Jour. Physiol., 1909, 39, p. 98.

<sup>5</sup> Biochem. Jour., 1914, 8, p. 70.

by Taylor and Adolph<sup>6</sup> and others that Benedict's method of estimating the amount of urea includes with the urea about 70-75% of the allantoin present. In the rabbit allantoin represents the chief end product of nucleic-acid metabolism, and we have considered the allantoin fraction obtained by difference between the two determinations of urea as an index of nucleic-acid metabolism and have used the uncorrected figures throughout. The tables therefore record the total nitrogen, the urea nitrogen (urease method), and the allantoin nitrogen (difference between the determinations by the Benedict and by the urease methods).

#### RESULTS AFTER INJECTION OF KILLED TYPHOID BACILLI

Table 1 gives the results in 3 of the rabbits injected with killed organisms. In each of these animals the leukocytic reaction was prompt and definite, and tho the counts were not made at very frequent intervals, yet those recorded show both a definite leukopenia and a subsequent leukocytosis. It is of course probable that the extreme in either was not obtained. In Rabbit 3 the high level of allantoin excretion is possibly to be explained as due to an infection with "snuffles," which was present in this rabbit to a mild degree, becoming more severe toward the end of the period. In none of these animals was there any marked change in the allantoin figures following the injection.

#### RESULTS AFTER INJECTION OF LIVING TYPHOID BACILLI

The results obtained after the injection of live organisms were much more variable. Examples are given in Table 2. In some instances (Rabbit 6) the allantoin fraction showed no more variation than in the animals injected with killed organisms. On the other hand, in some animals with no greater leukocytic reaction there occurred a marked rise in the total nitrogen figure, which was chiefly due to an enormous increase in the allantoin fraction. Thus, in Rabbit 4 the previously high allantoin figure was doubled the day following the injection and in Rabbit 5 the increase was from 42 to 448 mg. In the latter animal this increase did not appear until the day following the injection and we failed to obtain a count showing leukocytosis until 3 days later. It is probable, however, that the usual leukocytic crisis did occur and was missed as a result of the infrequency of the counts taken. This animal was never catheterized, but the increase in the allantoin figure is too great to be explained by any such factor. Rabbits 4 and 6 had been previously immunized with 3 injections of killed bacilli given at 2-day intervals in the hope of obtaining a greater

<sup>6</sup> Jour. Biol. Chem., 1914, 18, p. 521.

TABLE 1

ALLANTOIN EXCRETION IN RELATION TO LEUKOPENIA AND LEUKOCYTOSIS FOLLOWING THE INJECTION OF KILLED TYPHOID BACILLI INTO RABBITS

Day	Weight, Gm.	Urine, c.c.	Nitrogen			Leukocytes
			Total	Urea	Allantoin	
RABBIT 1 *						
1	....	95	.158	.136	.022	Before injection,† 5800 1 hour after injection, 2100 24 hours after injection, 24,000
2	....	115	.130	.092	.038	
3	....	105	.128	.098	.030	
4	....	98	.109	.098	.011	
5	1100	58	.075	.049	.026	
6	....	130	.125	.092	.033	
7	950	60	.055	.049	.006	
RABBIT 2						
1	....	155	.449	.299	.095	Before injection,† 15,900 1 hour after injection, 2400 26 hours after injection, 43,000 50 hours after injection, 13,800
2	....	160	.579	.326	.134	
3	....	140	.435	.272	.090	
4	....	158	.443	.250	.082	
5	1485	152	.457	.266	.096	
6	....	140	.370	.223	.072	
7	1430	Urine lost				
8	....	Urine lost				
9	1320	130	.481	.265	.070	
RABBIT 3						
1	1760	100	.575	.218	.299	Before injection,† 13,600 1 hour after injection, 4000 19 hours after injection, 50,000 24 hours after injection, 108,400 48 hours after injection, 28,200 72 hours after injection, 8100 Severe snuffles
2	1790	145	.554	.187	.281	
3	1790	150	.523	.229	.234	
4	1775	175	.484	.203	.203	
5	1735	180	.377	.140	.237	
6	1645	110	.671	.229	.327	
7	1635	145	.733	.300	.353	
8	1640	153	.751	.338	.320	
9	....	...	.....	.....	.....	

\* Not catheterized.

† Rabbits 1, 2, and 3 each received an injection of one-tenth slant of killed typhoid bacilli on the 5th day.

TABLE 2

ALLANTOIN EXCRETION IN RELATION TO LEUKOPENIA AND LEUKOCYTOSIS FOLLOWING THE  
INJECTION OF LIVING TYPHOID BACILLI INTO RABBITS

Day	Weight, Gm.	Urine, c.c.	Nitrogen			Leukocytes
			Total	Urea	Allantoin	

## RABBIT 4

1	....	130	.434	.130	.255	Before injection,† 10,500 1 hour after, 2700 21 hours after, 36,000 24 hours after, 11,300 48 hours after, 14,200
2	....	155	.528	.218	.251	
3	....	125	.577	.208	.304	
4	1665	170	.598	.213	.341	
5	....	200	.996	.333	.648	
6	....	180	.869	.348	.472	Diarrhea; W. B. C. 11,400
7	1510	150	.725	.229	.450	
8	1515	170	.803	.255	.509	
9	....	160	1.134	.442	.619	
10	....	190	1.053	.332	.643	
11	1430	82	.648	.224	.390	
12	1470	90	.434	.142	.261	
13	1520	90	.322	.111	.159	
14	1570	135	.252	.083	.143	
15	1560	162	.393	.104	.242	

## RABBIT 5 \*

1	....	...	.....	.....	.....	Before injection,† 7000 1 hour after, 3600 24 hours after, 6700 22,300
2	1280	75	.326	.207	.024	
3	....	80	.316	.234	.019	
4	....	50	.245	.141	.049	
5	....	80	.348	.246	.042	
6	....	100	.903	.370	.448	22,300
7	1140	45	.351	.190	.115	
8	....	100	1.100	.751	.244	
9	Sick—killed		.....	.....	.....	

## RABBIT 6

1	....	140	.382	.130	.156	Before injection,† 12,200 1 hour after, 2800 21 hours after, 33,300 24 hours after, 20,300 48 hours after, 11,700
2	....	125	.390	.187	.151	
3	....	130	.447	.156	.221	
4	1397	138	.432	.146	.178	
5	....	100	.426	.151	.144	
6	....	175	.426	.151	.179	48 hours after, 11,700
7	1320	80	.270	.078	.174	

\* Not catheterized.

† Rabbits 4, 5, and 6 each received injections of living typhoid bacilli on the 5th day, Rabbits 4 and 6 receiving one-tenth slant, and Rabbit 5, one-half slant. Rabbits 4 and 6 had previously been immunized with 3 doses of killed organisms at 2-day intervals.

leukocytosis, as described by Gay and Claypoole.<sup>7</sup> However, no hyperleukocytosis was obtained and these animals reacted similarly to the others.

#### DISCUSSION

Concerning the animals injected with dead organisms it must be concluded that no light is shed by this study either on the fate of the leukocytes driven into the internal circulation or on the relation of allantoin excretion to the leukopenia and leukocytosis brought about by such injections.

In the animals given injections of living organisms the leukocytic reactions, at least in the peripheral blood, are the same as when dead organisms are injected, so that an additional factor must be invoked to explain the sudden increase in the nitrogen metabolism and especially in the allantoin fraction. Two possibilities suggest themselves. First, there is a possibility that when live organisms are injected there occurs a destruction of leukocytes in the internal circulation as a result of their activity in taking up the injected bacilli and that this does not occur when heated cultures are employed. On the other hand, there is the second possibility that the changes in excretion are independent of the leukocytic reaction altogether and dependent on some other factor, such as fever, which in turn is induced only by the living organisms. This is made probable by the figures observed in a rabbit not detailed in this report which developed severe snuffles during a preliminary control period. This animal exhibited an abrupt rise of total nitrogen and of the allantoin fraction similar to that obtained following the injection of living organisms. It is impossible, however, to arrive at any positive conclusion on this point until further investigation shall have been made.

#### CONCLUSIONS

Injection of typhoid bacilli either living or dead into the peripheral circulation of the rabbit causes a leukopenia and subsequently a leukocytosis.

After injections of dead organisms there occurs no alteration in the nitrogenous excretion, as shown in determinations of total nitrogen, urea nitrogen, and allantoin nitrogen.

After injections of living organisms there frequently occurs an abrupt increase in nitrogenous excretion, which is chiefly due to a marked increase in the allantoin fraction.

<sup>7</sup> Arch. Int. Med., 1914, 14, p. 662.

# A LABORATORY INFECTION CAUSED BY A BOVINE STRAIN OF BACILLUS ENTERITIDIS \*

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Cases of meat poisoning caused by *Bacillus enteritidis* (Gärtner) or closely allied organisms are very rare in the United States. The rarity of such infections is surprising in the light of numerous observations showing that food-producing animals, as well as experimental animals, are not infrequently infected with these organisms. In recent years, for instance, paratyphoid organisms have been isolated from canary birds and horses, and *B. enteritidis* has been found present in guinea-pigs, rats, dogs (Torrey and Rahe<sup>1</sup>), and calves (Meyer, Traum, and Roadhouse<sup>2</sup>). All of these animals act, in various ways, as sources of infection for man, and in fact the history of meat-poisoning epidemics has shown that they coincide in a most remarkable manner with the consumption of meat from animals infected with septic diseases caused, for the most part, by bacteria which biologically are identical with those isolated from the gastro-intestinal canal of cases of meat poisoning in man. Workers have always been disappointed in not being able to demonstrate experimentally the pathogenicity for men of the animal strains of the paratyphoid-enteritidis group of bacteria, altho descriptions of a few human infections with mouse typhoid bacilli<sup>3</sup> have supplied some valuable information concerning the pathogenesis of paratyphoid organisms. In the light of the many undecided questions which such infections present for consideration, it seems worth while to give a description of an interesting laboratory infection which took place during an investigation of the etiology of infectious diarrhea of calves.

## THE CASE

*History.*—A young man, aged 26 years, was infected January 14 while assisting in the feeding of a calf with sterilized milk containing 25 c.c. of a 24-hour-old culture of the paracolon bacillus (*B. enteritidis*, Gärtner, Strain

\* Received for publication July 21, 1916.

<sup>1</sup> Jour. Med. Research, 1912, 27, p. 315.

<sup>2</sup> Jour. Am. Vet. Med. Assn., 1916, 49, p. 17.

<sup>3</sup> See the publications of Trommsdorff, München. Med. Wchnschr., 1903, 50, p. 2092; Meyer, Ibid., 1905, 52, p. 2261; Shibayama, Ibid., 1907, 54, p. 979; and Handson, Williams, and Klein, Brit. Med. Jour., 1908, 2, p. 1547.

1239), which had been isolated 30 days previously. The patient could not recall the manner in which he had contaminated his hands nor did he remember what precautions he had employed for disinfecting them before the evening meal.

From 10 to 12 hours later the patient was seized with severe abdominal cramps, nausea, and diarrhea. By this time he had passed from 3 to 4 semi-solid stools, and by the end of the first 20 hours he had passed 10. The nausea, abdominal cramps, and flatulence continued. The temperature at this time was 101 F., and the patient complained of loss of appetite, headache, and thirst. The tongue was coated, and there were marked fetor oris, and suppression of urine.

Forty-eight hours later the temperature fell to 99.9 F., but the patient still felt weak and depressed. Abdominal pain, tenesmus, and diarrhea continued. During the second 24 hours, he had passed 12 watery stools. These were deeply bile-stained, like rice soup in character, and contained greenish pellets of fecal matter. In the evening the patient took 5 grains of calomel. The diarrhea did not cease, the patient passing 10 more liquid stools in the subsequent 24 hours. These were light-yellow but contained very little mucus. In the evening the patient again took 5 grains of calomel.

On January 18 the patient's temperature again rose to 100 F. He felt weak and thirsty, with a peculiar desire for salt. On this day the patient had passed from 8 to 10 stools. The diarrhea persisted for 2 more days, during which he passed from 6 to 10 stools.

A week following the onset of his illness the patient felt well and ate his regular meals without any ill effect. So far as could be ascertained he had suffered from a chronic mucous colitis, which persisted more or less unaffected by the present illness, as was evidenced by the irregular elimination of mucous casts.

*Bacteriologic Examination.*—The relation of the gastro-intestinal disturbance to the feeding experiment was unsuspected until 4 days after the onset of the infection. At this time a solid particle of feces was enriched in bile broth and after 12 hours' incubation was plated on litmus-lactose agar. Numerous bluish transparent colonies developed, which were identified as *B. enteritidis* (Gärtner). The various subcultures obtained, behaved in every respect like Calf Strain 1239.<sup>2</sup> Its characteristic behavior in arabinose broth was noted; indeed, even the first 8 or 10 transplants failed to ferment this pentose. In the white colonies on solid arabinose endo medium there developed, in from about 5 to 7 days, red bud-like daughter colonies consisting of an arabinose "mutant."

The first few transplants of the bacillus were agglutinated only in a dilution of 1:6000 by a serum which agglutinated Strain 1239 in a dilution of 1:10,000. A sample of stool was plated on January 21 directly on litmus-lactose agar. Thirty-seven colonies of *B. enteritidis* and 30 colonies of lactose-fermenting organisms were found in 1 loopful of liquid stool. Stool samples taken on the following days gave positive findings of paracolon bacilli: January 18, 20, 21, 23, 25, 26, 28, 30, and February 1. The samples taken on February 3, 5, 10, May 10, and June 19, were found free of paracolon bacilli, even after an enrichment in bile and in malachite green broth. A large mucous cast eliminated in June proved bacteriologically to be free from *B. enteritidis*.

The pathogenicity of the strain isolated from the laboratory infection is, in some respects, interesting. Mice fed with broth directly by placing 1 drop of a 24-hour-old broth culture on the tongue, either survived or died, with all the lesions of an infection with *B. typhi-murium*, in from 8 to 15 days after

feeding of the bacteria. Mice fed however with meat soiled with a few drops of a suspension of *B. enteritidis*, succumbed regularly to the feeding infection in from 4 to 10 days, the anatomic lesions consisting of a hemorrhagic colitis, splenic tumor, and liver necroses. In a few instances some of the control mice acquired the infection from infected animals occupying the same cage.

*Serologic Examination.*—The serum of the patient agglutinated neither the typhoid bacillus nor the paracolon bacillus isolated from his stools 6 days after the onset of the gastro-intestinal infection.

On February 3 the serum of the patient was tested against various intestinal organisms, and agglutination occurred in a dilution of 1:1280 only with the paracolon organisms isolated from the patient and with those previously cultured from the calves. No agglutination with one typhoid bacillus strain was obtained.

TABLE 1  
RESULTS OF AGGLUTINATION TESTS IN A CASE OF INFECTION WITH *B. ENTERITIDIS*

Antisera	Bacillus of Patient	<i>B. Enteritidis</i> (Strassburg)	<i>B. Enteritidis</i> (A. M. N. H.)
<i>B. typhosus</i> (Cross).....	1:200	1:40	1:60
<i>B. paratyphosus</i> A (polyvalent).....	0	0	1:200
<i>B. paratyphosus</i> A (Strassburg).....	0	0	1:20
<i>B. paratyphosus</i> B (polyvalent, Nos. 4 and 5).....	0	1:2000	1:1000
<i>B. paratyphosus</i> B (homo, Strassburg).....	0	0	0
<i>B. suipestifer</i> (Strassburg).....	0	0	1:100
<i>B. suipestifer</i> V.....	0	1:20	1:20
<i>B. suipestifer</i> (Voldagsen).....	0	0	0
<i>B. suipestifer</i> (typhi-suis).....	0	0	0
<i>B. abortivus-equinarius</i> .....	0	0	0
<i>B. enteritidis</i> (A. M. N. H.).....	1:10,000	1:10,000	1:10,000
<i>B. enteritidis</i> (Strassburg).....	1:20,000	1:40,000	1:10,000
<i>B. enteritidis</i> (guinea-pig, Plotz No. 4).....	1:10,000		
<i>B. enteritidis</i> (No. 5, calf strain 1239).....	1:6000		
<i>B. enteritidis</i> (No. 18).....	1:2000		1:6000
<i>B. typhi-murium</i> (Loeffler No. 1).....	1:10,000	1:8000	1:10,000

Monovalent and polyvalent sera prepared with well-known organisms of the typhoid-paratyphoid groups gave, with the paracolon bacillus of the patient, reactions which are summarized in Table 1. For comparison, 2 other typical strains of *B. enteritidis* are included in the table. The absence of co-agglutination of the patient's strain by paratyphoid sera is interesting in many respects.

Through the careful investigations of Poels and Jensen<sup>4</sup> it has become established that the so-called paracolon bacilli isolated from calves suffering with infectious diarrhea are related both serologically and biochemically to *B. enteritidis* (Gärtner) and to the ratin bacillus. These findings naturally suggest the possibility that these organisms may also play a part in the etiology of certain enteric fevers in man. The meagerness of studies in this connection may be attributed to the fact that these organisms have been considered to be pathogenic only for man, and that food-producing animals have been thought to become

<sup>4</sup> Kolle and Wassermann's Handb. d. pathogen. Microorganismen, 1913, 6, p. 126.

only accidentally infected with such organisms. But up to within recent years the possibility of the existence of a reverse condition was not entertained. This was largely because of the failure to produce in large animals a paratyphoid-like disease by the inoculation of such organisms found pathogenic for man.

However, the facts concerning the distribution and prevalence of bacteria belonging to the paratyphoid-enteritidis group which have accumulated in the last few years, have led many hygienists to express the belief that all cases of meat poisoning are directly due to intravital contamination of the meat by such bacteria.

Furthermore, carefully conducted inquiries into recent outbreaks of meat poisoning have again failed completely to support this contention. In this connection it is remarkable that very few cases of meat poisoning are reported in the United States, where hog cholera is very prevalent. In this disease of swine, secondary invaders of the paratyphoid group are always present and are doubtless being taken into the human intestinal tract without ill effect. Again, the occurrence of infection in calves with organisms like *B. enteritidis* has been reported in California, and yet no epidemics of meat poisoning have been found traceable to veal infected with *B. enteritidis*.

This may be explained by the fact that in the United States, unlike Belgium and Germany, which, according to Sacquépée,<sup>5</sup> are "*les terrains de predilection des intoxications alimentaires*," no emergency slaughtering of diseased animals is practiced, and the meat of such animals is rarely consumed. Moreover, the dangers from infected veal are reduced by the strict, tho wasteful, regulations adopted by the various states in the inspection of bob veal and meat in general. Infections from postmortem-contaminated meat are also reduced on account of the limited use of minced, uncooked, or half-cooked meat and the probable absence of a sufficiently large number of human carriers. The yearly increasing improvements in dairy inspection and pasteurization of milk lessen the possibility of infections by this channel.

Probably all these conditions are only in part responsible for the absence of infections with *B. enteritidis* in the United States. Thus far no proof has been brought forward to show with any certainty that the paracolon bacilli or *B. enteritidis* isolated from diseases of calves are always pathogenic for man and, therefore, always capable of causing meat poisoning. In the state of California paracolon-bacillus infections of calves are not rare; thus far, only one epidemic

<sup>5</sup> *Les empoisonnements alimentaires*, 1909, p. 12.

of meat poisoning is on record (Hogan<sup>6</sup>), and this has not been subjected to a rigorous bacteriologic investigation.

In explanation of these facts two possibilities must be entertained: In the first place, the various strains of *B. enteritidis* may not be identical in their behavior towards man, or it is not unlikely that some predisposing factors, so far unknown, are necessary for successful infections. Secondly, it is possible that epidemics of meat poisoning caused by *B. enteritidis* or paracolon bacilli do occur, but that they are of such mild character as to be rarely brought to public attention.

The first possibility is apparently well supported by the observations of Wiemann<sup>7</sup> and Rimpau,<sup>8</sup> who state that veal of animals from which they had isolated paracolon bacilli had been eaten without causing the slightest harm. In support of this, Wiemann reports the following instance: On a farm, over 60 calves suffering from paracolon bacillosis were used as food either after slaughter or after death, and yet no infection resulted.

That the second explanation also has some facts to support it is shown by a statement of Christiansen.<sup>9</sup> In Denmark, paracolon bacillosis of calves is one of the greatest scourges of the livestock industry, and yet extensive reports of epidemics of meat poisoning are unknown.

Referring to this point Christiansen<sup>9</sup> in his article on paracolon bacillosis states:

"Even if very serious cases of poisoning are rarely substantiated—which does not mean that they rarely occur—still we have lately seen a case here of mild meat poisoning. Thus in the last few years wholesale cases of meat poisoning have occurred at the hospitals of Copenhagen; fortunately these cases have been of a mild nature in that the disease has been limited to thin purgation a few times together with stomach cramps and in the worst cases vomiting and diarrhea for a day. These cases have always occurred after the eating of a meat course, which in almost all cases, has been of veal. But this does not indicate that paracolon bacillosis caused them, for investigations in regard to the etiology of the cases are not at hand, but they show at any rate that cases of meat poisoning, especially such as are occasioned by the eating of veal, are not so rare, and like cases could very well occur in greater number in the country, without being brought to the knowledge of the public if peculiar circumstances connected with their appearance, in hospitals and like institutions, had but made them better known. It is therefore not right to say that meat poisoning rarely occurs here in this country, nor is it right to use this as an argument for the safety of man against the paracolon

<sup>6</sup> Bull. California State Board of Health, 1908, 4, p. 67.

<sup>7</sup> Thesis, University of Bern, 1909, p. 24.

<sup>8</sup> Klin. Jahrb., 1911, 22, p. 145. Arch. f. Hyg., 1912, 76, p. 9.

<sup>9</sup> Rep. Serum Lab., Roy. Vet. and Agr. High School, Copenhagen, 1915, 35, pp. 4, 73.

bacilli." (I am indebted to Miss Louise H. Madsen for the translation of the Danish text.)

The necessity of a careful bacteriologic investigation of every gastro-intestinal infection is again suggested by these observations, and the collection of all the data concerning the behavior of paracolon bacilli in man is made imperative.

#### DISCUSSION

The detailed description of a laboratory infection with *B. enteritidis* isolated from the blood stream of a calf suffering from infectious diarrhea demonstrates the fact that such strains can acquire pathogenicity for man. The course of the infection was severe, probably due to predisposing conditions in the intestinal tract of the patient. This predisposition in form of a mucous colitis is doubtless of considerable importance, since at least 5 other members of the laboratory staff handled, in a very careless manner, feces, organs, and cultures richly impregnated with these paracolon bacilli, and yet no additional infection was brought to our attention. Unfortunately specimens were not obtainable from all the men who handled material contaminated with paracolon bacilli. It would have been interesting to determine the possible occurrence of individuals who could temporarily eliminate the bacilli, as Conradi<sup>10</sup> and Rimpau<sup>8</sup> were able to show on several occasions. Thus, in the famous meat-poisoning epidemic of St. Johann, numerous persons who had eaten infected meat remained in perfect health and yet eliminated Gärtner bacilli, according to Rimpau. That *B. enteritidis* of animal origin is only pathogenic in man when particularly favorable conditions obtain in the internal tract for their localization, is quite clear from numerous statements and from the evidence presented; it is therefore unnecessary to assume that some strains are more virulent than others. Naturally, passage through man will enhance their pathogenic properties for man, and contact infection, in which milk and other foodstuffs can act as vehicles, will result.

It would appear from our observations that in this instance the introduction of heat-resistant toxins together with the bacteria did not play a very important part in the infection. The toxin produced in the broth was considerably diluted with milk and it is proper to assume that the bacteria alone were taken into the intestinal tract of the patient.

<sup>10</sup> Klin. Jahrb., 1909, 21, p. 421.

According to the experiments on mice the addition of meat to the paracolon bacilli enhances the chances for a successful infection. Probably the toxins which are more extensively produced in meat than in other media, as well as the catarrhal inflammation which results from a diet of raw meat in mice, may be the predisposing factors for the infection.

The virulence of the paracolon organisms was also not particularly high for calves; one feeding experiment did not result in the death of the animal and it was noted that several calves recovered spontaneously from contact infection contracted under natural conditions.

The course of the laboratory infection corresponded well with the typical gastro-intestinal form of paratyphoid infection. Complete recovery resulted in less than 2 weeks. The causative micro-organisms disappeared entirely from the intestinal canal in 20 days after the infection. Repeated stool and numerous cast-examinations gave always negative results after February 3. This observation confirms the observations by Rimpau and others in numerous epidemics, that chronic carriers of *B. enteritidis* do not develop as the result of meat poisoning.

The positive agglutination reaction with the serum of the patient is further proof of the etiologic relation of the calf strain to the infection. The observation that the serum failed to co-agglutinate two strains of *B. typhosus* is, in the light of Langkau's<sup>11</sup> work, very suggestive. Bacteria of the Gärtner group always have the tendency to be co-agglutinated by a typhoid serum, even in high dilutions, and in contrast with sera in paratyphoid infections, sera of patients infected with *B. enteritidis* in most instances agglutinate *B. typhosus* (Rimpau<sup>12</sup>). Furthermore, Langkau was able to show that paracolon strains from calves are not co-agglutinated by typhoid- or paratyphoid-immune sera, a condition which is always characteristic of strains of *B. enteritidis* from man, and that this behavior gives a means of differentiating the various types of *B. enteritidis*. The agglutination tests in Table 1 apparently support his findings with the exception that a typhoid serum co-agglutinated the human as well as the calf strain (not tabulated, for the sake of brevity) of *B. enteritidis*. On the other hand, the paratyphoid and suipestifer sera did not agglutinate the paracolon strains.

<sup>11</sup> Thesis, Leipzig, 1909.

<sup>12</sup> München. med. Wehnschr., 1909, 56, p. 1843.

So far as we are aware, this is the first instance reported in the literature in which it has been demonstrated that a strain of *B. enteritidis* pathogenic for an animal can, in a particularly predisposed human subject, cause the typical symptoms of meat poisoning. Such susceptibility of the individual doubtless plays an important rôle under practical epidemiologic conditions of meat poisoning in transforming the strain of paracolon bacilli pathogenic for animals into one pathogenic for man, for as a rule the strains of *B. enteritidis* isolated from calves, guinea-pigs, and dogs are of low virulence and, according to a few scattered observations, do not possess the high pathogenicity for man characteristic of the true meat-poisoning organisms.

#### CONCLUSIONS

This paper presents clinical, bacteriologic and serologic observations of an accidental laboratory infection. A young man who apparently was predisposed to the infection on account of a chronic mucous colitis developed a severe gastro-interitis 10 hours after having handled a bottle of sterilized milk which was artificially contaminated with a culture of *B. enteritidis* (Gärtner). The strain responsible for the infection had been isolated from the heart blood of a calf which had succumbed to infectious diarrhea. Evidence is presented to show that a recently isolated strain of *B. enteritidis* pathogenic for animals may differ from a strain pathogenic for man in its inability to be co-agglutinated by paratyphoid or suipestifer sera.

# FURTHER STUDIES ON THE VIRULENT SALT SOLUTION USED IN THE PRODUCTION OF HOG-CHOLERA SERUM \*

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No accurate method of standardizing hog-cholera virus has been found. Certain strains of it, however, can usually be developed to such a point as to yield fairly uniform results. In the production of hog-cholera serum at this station, the original (Ames, Iowa, 1908) Dorset-Niles strain of virus is used, which, when injected intramuscularly in doses of from 3 to 5 c.c., almost invariably produces typical symptoms of hog cholera and death in susceptible pigs, averaging 100 lb. each in weight, in from 6 to 7 days. When kept at this virulence, it has never failed, by subcutaneous methods, to produce potent serum. In studying the nature of virulent salt solution, I have accepted this known strain of virulent blood as a standard of comparison, and have endeavored accurately to compare virulent salt solution with virulent blood from the same virus pigs.

Graham and Himmelberger<sup>1</sup> found virulent salt solution efficient in hyperimmunization by the intravenous method when mixed with virulent blood. However, their work gives no information as to the virulence of salt solution, since the two viruses were used together and in each case 7 c.c. of the mixture to the pound of body weight.

Craig<sup>2</sup> found that 10 pigs inoculated intramuscularly with 2 c.c. each of virulent salt solution lived an average period of 13 days. Nine pigs injected with 2-c.c. doses of virulent blood from the same source lived an average period of 9 days. No mention is made of how long the salt solution remained in the abdominal cavities of the virus pigs nor of the amount injected into them. From these tests it appears that the salt solution used was not as virulent as virulent blood.

Such tests, by direct subcutaneous or intramuscular inoculation, do not appear to be an accurate means of standardizing virulent salt

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<sup>1</sup> Jour. Infect. Dis., 1916, 18, p. 118.

<sup>2</sup> Bull. Purdue Univ. Agr. Exper. Sta. No. 173, 1914, p. 446.

solution, as the virulent salt solution is absorbed more readily and more completely than the virulent blood. Direct intravenous inoculations perhaps would give more accurate results. It also seems reasonable that hog-cholera virus could be accurately tested by using it to hyperimmunize by the intravenous method and then testing the serum secured against a known strain of virus.

In my previous work<sup>3</sup> I found that 6-hour 25-c.c. virulent salt solution (salt solution injected into the abdominal cavity of virus pigs at the rate of 25 c.c. to the pound of body weight and allowed to remain for 6 hours) by the subcutaneous methods would uniformly produce serum of high potency. In the hyperimmunization of several hundred pigs by the subcutaneous methods with mixtures usually of about equal parts of virulent blood and virulent salt solution, I have produced a very potent serum in every case. In so far as the production of hog-cholera serum by the subcutaneous methods is concerned, we may accept virulent salt solution as equal to virulent blood as a virus for hyperimmunization. However, this does not mean that 6-hour 25-c.c. virulent salt solution is as virulent as virulent blood. The absorption of any liquid injected subcutaneously or intramuscularly varies according to the density as well as the bacterial and chemical contents of the liquid. The use of virulent salt solution has reduced to a minimum the abscesses that follow subcutaneous injections of straight virulent blood. In many cases these local abscesses no doubt mean a loss of one-half the virulent blood injected, the blood becoming encysted at the point of inoculation and so shut off from the absorptive system. Therefore, it appears that if virulent salt solution were only one-half as virulent as virulent blood, it might produce serum of potency equal to that produced by virulent blood by the subcutaneous methods, on account of its being more completely absorbed. When used together with virulent blood, the efficiency of the latter is increased because, the density being lowered, the degree of absorption is increased.

Before making further biologic tests of virulent salt solution it was thought advisable to learn something of its chemical and bacteriologic contents as compared with those of virulent blood. With the assistance of Mr. Herman Waagbo, I have made analyses of the two viruses from 25 virus pigs, the results of which are given in Table 1.

The data show that virulent salt solution contains very small percentages of fibrin and protein as compared with virulent blood and a much smaller number of colonies of *B. cholera-suis*, altho this organ-

<sup>3</sup> Jour. Infect. Dis., 1913, 12, p. 335.

TABLE 1

COMPARATIVE ANALYSIS OF VIRULENT SALT SOLUTION AND VIRULENT BLOOD FROM THE SAME PIGS

Number of Pigs	Rate of Injection, c.c. per lb.	Time in Abdominal Cavity hr.	Percentage Recovered	Average Percentage of Fibrin		Average Percentage of Protein		Colonies B. Cholera-Suis per c.c.	
				Virulent Salt Solution	Virulent Blood	Virulent Salt Solution	Virulent Blood	Virulent Salt Solution	Virulent Blood
5	25	4	68.6	.058	1.09	.45	15.55	1275	15,350
5	25	5	68.7	.041	1.49	.59	17.41	41	63,139
5	25	6	61.4	.067	0.62	.99	17.75	3	6,468
5	25	7	48.0	.077	1.16	.65	16.18	92	223,330
5	25	8	53.0	.082	1.01	.99	17.68	538	2,343

ism was uniformly present. The percentages of fibrin and protein in the salt solution increased with the time it remained in the abdominal cavity.

Since the potency of hog-cholera serum depends largely on the virulence of the virus used in its production, it seemed that an accurate comparison of virulent salt solution and virulent blood through the hyperimmune, using the intravenous method, would give us some knowledge of the virulence of salt solution. With this in view I drew 6-hour 25-c.c. virulent salt solution and virulent blood from the same virus pigs and used these viruses as "straight virulent salt solution" and "straight virulent blood" in hyperimmunizing 6 pigs by the intravenous method. Three were injected with virulent salt solution at the rate of 6 c.c. to the pound of body weight, and 3 at the same rate with virulent blood. One of the hyperimmunes receiving blood died soon after the injection. The other two were carried through the experi-

TABLE 2

RECORD OF TEST

Pig	Weight, lb.	Serum Injected c.c.	Virus Injected c.c.	Results
953	80	25	2	Died within 10 days; typical cholera lesions
954	90	20	2	
955	75	15	2	
956	90	25	2	Died within 8 days; typical cholera lesions
957	100	20	2	
958	75	15	2	
959	95	None	2	Lived
960	90	None	2	

ment together with the three that had received the salt solution. The sera from the latter were mixed together and likewise those from the two hyperimmunized with virulent blood. The resultant mixed sera were tested on 8 susceptible pigs as indicated in Table 2.

The results of this test indicate that virulent salt solution is not nearly so virulent as virulent blood, and that while it is efficient in hyperimmunizing by the subcutaneous method, it would no doubt be impractical in the intravenous method because of the difficulty of injecting enough of it intravenously to produce very potent serum. Even if it is used in mixture with virulent blood, the amount of the mixture required to produce good serum would be unusually high.

# THE ETIOLOGY OF FIBROMYOMA OF THE UTERUS \*

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The etiology of fibromyoma of the uterus has been studied from numerous angles, but we have nothing more than theories to account for the development of these tumors. The most generally accepted explanations of the pathogenesis of fibroids are Conheim's inclusion theory, upheld by Ricklinghausen, by Wolff and Müller, and by Ribbert; infection, suggested by Kolmann and by Galippi; Virchow's theory of irritation, the causes of irritation being as yet unknown; blood-vessel origin, upheld by Klebs, Gottschalk, Lubarsch, Keiffer, Pillet, and Costes—a favorite theory; and finally, the ovarian theory of Hegar, Campbell, and Seitz, who hold that disturbed internal secretion stimulates tumor growth.

Meyer,<sup>1</sup> in a study of the histogenesis of myoma, found total absence of vessels in numerous small myomas, and is convinced that fibroids do not arise from blood-vessel walls. He considers misplaced tissue and uterine affections with irritation highly important in the etiology.

Heimann,<sup>2</sup> in a study of the minute anatomy of fibroids, also found an absence of blood-vessel connection with the small tumors. In sections through sclerotic uteri without macroscopic fibroids he discovered rudimentary small tumors arising from muscle fibers.

Much stress has been placed on irritation as a factor in the pathogenesis of fibroids, yet there is a total absence of bacteriologic work in this field. It seemed to me desirable that this subject be investigated, and with the employment of a special technic it has been possible satisfactorily to proceed with such a study. Since Jan. 28, 1914, all available uterine fibroids have been examined with the object of throwing more light on the relation which infection may bear to their development.

\* Received for publication July 22, 1916.

<sup>1</sup> Veit's Handb. d. Gyn., 1906, 1, p. 457.

<sup>2</sup> Ztsch. f. Geburtsh. u. Gynäk., 1912, 69, p. 719.

In most instances the material has been under control from the moment of opening the abdomen. This is of advantage, for it makes possible the observance of unusual care in securing uncontaminated material; also, in the interpretation of certain lesions, personal observation of the unmanipulated tissues proves to be of considerable help.

After elimination of fibromyomas undoubtedly contaminated or otherwise unsatisfactory (this necessarily excluding cultures from material from patients with recent pelvic infection), there is left a series of 80 cases.

Evidence suggestive of previous pelvic infection, old enough to merit consideration as a possible factor in the etiology of tumor growth, was found 14 times—slightly less than 18%. This is a smaller percentage of chronic pelvic infection than is ordinarily found in patients with fibromyoma.

In gross characteristics the growths revealed only such variations as we should expect to encounter in this number of cases. Histologic preparations presented no evidence of inflammatory changes. Stains for bacteria all were negative.

Disturbances elsewhere in the body, notably infections, were looked for in these patients as evidence that fibroids develop more frequently in persons with focal infections, and also for the purpose of noting whether systemic disturbances in fibromyomatous patients can be ascribed to long-drawn-out low-grade infections residing in these tumors. As would be anticipated, focal infections were often present, but no causal relation between them and the development of fibroids was evident. Systemic disturbances seemed at times out of proportion to the direct effects of pressure, hemorrhage, and other local conditions, but whether chiefly due to absorption of tumor material or in part ascribable to infectious processes, is purely conjectural. New and Wolff,<sup>3</sup> in an experimental and anatomic study of heart lesions in myoma cases, concluded that a specific myoma heart does not exist; the chief changes are due to anemia, but there is also a "tumor consumption heart."

#### TECHNIC

Skin clips with protective pads, also the resterilization of each instrument immediately after use, contributed to minimize contamination during operation. The uteri removed were at once wrapped in sterile towels, placed in the ice chest, and cultured as soon as practicable.

With all in readiness the towels were opened, the uterine surface was seared with a hot spatula, and portions of the tumors excised. Separate instruments were employed for the removal of each piece of tissue.

<sup>3</sup> München. med. Wehnschr., 1912, 59, p. 72.

A sterile meat crusher, provided with a cloth covering to prevent air contamination, was used to express the fibroid juice and fine shreds of tumor tissue. This technic afforded a satisfactory asepsis and yielded much tissue extract. Similar pieces from the same locality were ground in sand by means of a pestle and mortar enclosed in a sterile cloth sack provided with a window (Rosenow). The latter procedure is not so eminently fitted for fibroid work because of the frequent toughness of the tissues.

In cases of succulent tumors, media were inoculated with the juice which exuded from the cut surfaces.

*Media.*—The concentrated turbid blood-stained fluid was clarified through mixture with broth or ascites broth and was then introduced into a sufficient number of culture tubes to insure moderately transparent media. Aerobic and anaerobic blood-agar slants, ascites broth, also ascites-agar shake cultures of varied reaction, some sugar-free and others containing 1% dextrose, were the most used in attempts to obtain bacterial growth. The usual variety of media were employed in subculture for purposes of identification.

#### RESULTS

As stated, some cases were eliminated because of obviously undesirable material or unsatisfactory technic. Eighty remained.

No growth was obtained from the fibromyomas of 45 patients.

Twenty-two more, virtually belong in the same group. A few of these contained occasional cloudlike spreading colonies of a contaminating gram-negative bacillus. A gram-negative thin bacillus, 2 to 3 microns in length, was another invader. Scattered staphylococcus colonies or surface contamination appeared in some.

Thirteen cases with positive findings were left for consideration. The detailed results obtained from them are shown in the accompanying table.

Diphtheroid bacilli were found in 3 cases (6, 9, and 11). In Case 6, there were many colonies of diphtheroid bacilli in each of several culture tubes. The material obtained from Case 9 was sufficient for only 2 shake cultures, but each contained a pure growth of half a dozen colonies. From Case 11, considerable numbers of diphtheroid bacilli and numerous colonies of staphylococci were isolated.

In one other instance (Case 1) were found many staphylococcus colonies of such type that their presence suggested infection.

Aerobic streptococci were found 3 times, in Cases 7, 12, and 13. Innumerable colonies of nonhemolyzing long-chained streptococci developed in cultures from the endometrium and from the fibroid of Case 7. This tumor was of an infiltrating type, without clearly defined capsule. In the other two instances cultures revealed only scattered colonies of nonhemolyzing streptococci, once in association with gram-positive aerobic bacilli.

Anaerobic streptococci appeared once (Case 4). From 4 to 10 colonies were present in pure growth in each of several tubes.

Gram-positive small aerobic bacilli occurred in cultures twice. In Case 6, from 1 to 3 colonies were present in each of several culture tubes. A like growth was obtained from the endometrium. In the other instance, Case 13, in addition to several colonies of this organism, aerobic streptococci were also found.

Gram-positive diplococci were found once. The material employed consisted of several small fibroids, firmly embedded in the uterine wall, and possessed of ill-defined capsules. Each of 5 culture tubes contained 1 colony of gram-positive diplococci. These failed to form chains or to reveal characteristics of staphylococci in subcultures.

Colonies of gram-positive small paired bacilli developed twice. The presence of staphylococci in the first case renders these findings less valuable. In the other case, in addition to several colonies of this organism in each culture tube, a similar, but gram-negative, bacillus also developed. The latter organism appeared in no other instance.

*Bacillus welchii* grew freely in cultures from 1 case. In the same set of culture tubes there developed a knobbed gram-positive anaerobic bacillus.

As seen from the table, other pelvic lesions were present in 5 cases, in 3 of which they were definitely ascribable to infectious processes. The material from 2 of these was available for grinding, but growths were not obtained.

Whenever possible, the endometrium was examined for organisms throughout the series. In 13 cases of fibroids with bacteriologic finding, an attempt was made 6 times to procure growth from the endometrium. Cultures were obtained from 2, Cases 6 and 7. In these were found the respective organisms which were present in the fibroid cultures; namely, gram-positive bacilli and nonhemolyzing streptococci.

A number of the organisms isolated were tested for virulence. Intravenous injection of rabbits and intraperitoneal inoculation of guinea-pigs failed to reveal toxic effects, and autopsies were negative. Altho fresh subcultures were always used for animals, due allowance should be made for the time the organisms were grown on laboratory media; in some instances this amounted to many days.

Inoculation of the uteri and pelvic tissues of rabbits with the isolated strains of diphtheroid bacilli and streptococci failed to produce signs of tumor growth.

## ORGANISMS FOUND IN CULTURES FROM 13 CASES OF FIBROMYOMA SELECTED FROM A SERIES OF 80 CASES

Case	Staphylococcus	Diphtheroid Bacillus	Gram-positive Diplococcus	Aerobic Streptococcus	Anaerobic Streptococcus	Gram-positive Small Paired Bacillus
1	+	—	—	—	—	+
2	—	—	—	—	—	+
3	—	—	—	—	—	—
4	—	—	—	—	+	—
5	—	+	—	—	—	—
6	—	—	—	—	—	—
7	—	—	—	+	—	—
8	—	—	+	—	—	—
9	—	+	—	—	—	—
10	—	—	—	—	—	—
11	+	+	—	—	—	—
12	—	—	—	+	—	—
13	—	—	—	+	—	—
Total	2	3	2	3	1	2

## SUMMARY

Attempts to secure cultures were made with selected material from 80 patients with uterine fibroids. This material consisted of juices obtained by means of a meat crusher, and by grinding.

Forty-five specimens yielded no growth and 22 more were essentially negative.

Thirteen gave positive findings. In 6 of these the endometrium was studied with positive results in 2 cases, and negative results in 4.

In 2 instances in which the patients had suffered from previous pelvic infection the diseased tissues were ground and cultured in a search for growth similar to that obtained from the fibroids. The media remained sterile.

Diphtheroid bacilli, streptococci, and small gram-positive bacilli were most often found. None of these exhibited virulence for animals. There was no tendency to produce new growth in the uteri and other pelvic organs of animals inoculated.

## DISCUSSION

A causal relationship between bacterial invasion of the uterus and the development of fibroid tumors is not evident; the small percentage of cases which yielded growth, the considerable variety of the bacteria isolated, and the lack of pathogenicity for inoculated animals, permit no other deduction. These results, it may be stated, are not in accord with the impression which I have received from clinical experience.

ORGANISMS FOUND IN CULTURES FROM 13 CASES OF FIBROMYOMA SELECTED FROM A SERIES OF 80 CASES—*Continued*

Gram-positive Small Aerobic Bacillus	Gram-negative Minute Aerobic Bacillus	Bacillus Welchii	Anaerobic Gram-positive Small Bacillus	Knobbed Gram-positive Anaerobic Bacillus	Complications
—	—	—	—	—	Cyst, salpingitis, adhesions
—	+	—	—	—	
—	—	+	—	+	
—	—	—	—	—	Infected (?) edematous uterus
—	—	—	—	—	
+	—	—	—	—	
—	—	—	—	—	Chronic metritis
—	—	—	—	—	Papilloma ovary
—	—	—	+	—	
—	—	—	—	—	
—	—	—	—	—	Carcinoma cervix
+	—	—	—	—	
2	1	1	1	1	

There arises another question of some interest; namely, the significance which attaches to the occurrence of growth in 13 of the 80 cases studied. It seems improbable that technical errors are responsible for the essentially pure cultures obtained, yet such a possibility must be frankly admitted. A not illogical assumption is that bacteria lie inactive in various body tissues. This is an explanation which strongly appeals to me. Tubercle bacilli may long remain dormant in the lungs; typhoid "carriers" harbor their infections for periods of years; focal infections in bones, and probably also in kidneys, in lymph glands, and in the prostate, remain indefinitely. The European war has demonstrated that infection flares up on reamputation of limbs many months after apparent healing of the old stump.

In inactive tissues such as fibroid tumors, chronically enlarged breasts, and certain other resting organs, we are not accustomed to think that bacteria remain dormant. Perhaps these tissues, poor in phagocytic cells and protective leukocytes, are not so free from bacterial invasion as has been generally assumed.

## A CASE OF ANTHRAX \*

### PLATE 17

G. G. REINLE AND R. A. ARCHIBALD

*From the Research Department of the Western Laboratories, Oakland, California*

As anthrax in man is of rather rare occurrence, the following case is deemed worthy of report.

On June 3, 1916, Dr. L. A. Covel, a veterinarian, examined a cow that had died with clinical manifestations of anthrax. An ear removed from the cow for bacteriologic examination, revealed in smears and cultures the presence of *Bacillus anthracis*. Three days later Dr. Covel observed 4 small macules about the size of flea bites, and somewhat resembling them, on his right forearm. These eruptions were ushered in with a sensation of local burning and itching. In a few days they began to take on the characteristics of malignant pustules. The lesions, which were originally flat, began gradually to increase in size, and to project above the surface of the skin in the form of dark-purplish papules. At about this time a fifth pustule appeared and the whole arm commenced to swell.

On June 9 he placed himself under our care. The lesions, some of which were vesicular and some pustular in character, were immediately opened and a small quantity of seropurulent fluid discharged. Smears and cultures from each of the pustules showed large numbers of bacilli morphologically similar to *B. anthracis*. A diagnosis of anthrax was made, which was later confirmed by the cultures. The patient was removed to a hospital and immediately given 20 c.c. of antianthrax serum fresh from a hyperimmunized horse. The temperature at the time of admission to the hospital was 100.2, pulse 90. The arm was soaked in hot bichlorid solution, and then a Bier bandage was applied.

June 10.—Antianthrax serum was administered (25 c.c.), and the bichlorid soaking and Bier bandage were continued at intervals. The patient complained of backache and soreness in the extremities like that in an attack of grippe. The pustules at this time had assumed an appearance very characteristic of malignant pustules; that is, the central portion of the lesions was composed of dark-brownish, almost black, necrosed tissue, which made up about one-third of the entire lesion and was surrounded by a deep-red border, the whole being raised considerably above the surface of the skin.

Temperature: 7:30 a. m. 101, pulse 78; 5:30 p. m. 101.6, pulse 80; 10:45 p. m. 102, pulse 78.

Blood count: white cells 14,800; large mononuclears 3%; small mononuclears 7%; polymorphonuclears 89%; red cells 4,390,000; basophils 1%; hemoglobin 80%; color index 0.9+.

June 11.—Antianthrax serum (28 c.c.) was administered. Backache somewhat relieved. Inclined to sleep all the time. Pustules assuming a blackish appearance with well-defined borders. Arm still badly swollen, probably as a result in part of the Bier bandage.

\* Received for publication July 26, 1916.

Temperature: 7:30 a. m. 102.4, pulse 76; 12:15 p. m. 103, pulse 86; 3:45 p. m. 102, pulse 84.

Blood count: white cells 14,800; large mononuclears 4%; small mononuclears 5%; polymorphonuclears 90%; red cells 4,000,000; basophils 1%; hemoglobin 80%; color index 1.

June 12.—Antianthrax serum (30 c.c.) administered; bichlorid soaking continued. Feeling better; no backache and no pain in extremities; complained of cold feet and severe headache at all times. Pustules had become blacker and slightly raised. Arm still swollen.

Temperature: 7:30 a. m. 99.4, pulse 84; 1:00 p. m. 100.6, pulse 86; 3:45 p. m. 100.6, pulse 88.

Urine showed slight trace of albumin.

June 13.—Antianthrax serum (30 c.c.) administered. Patient much better. Pustules were still rising and a well-marked bright-reddish zone had developed about the periphery of the lesions. Swelling in arm not so marked.

Temperature: 7 a. m. 98.6, pulse 70; 1 p. m. 98.6, pulse 84; 4 p. m. 98, pulse 80.

Blood count: white cells 14,200; large mononuclears 7%; small mononuclears 11%; polymorphonuclears 80.5%; eosinophils 1.5%; red cells 4,520,000; hemoglobin 80%; color index 0.8 +.

June 14.—No serum given. Pustules at this time showed a black center surrounded by a white border and outside this an intensely red zone. Swelling on arm receding. Photographs in natural colors taken.

Temperature: 7 a. m. 97.6, pulse 68; 12 m. 97, pulse 68; 4 p. m. 97.6, pulse 68.

Blood count: white cells 11,600; large mononuclears 2.5%; small mononuclears 13%; polymorphonuclears 82%; eosinophils 2%; basophils 0.5%; red cells 4,310,000; hemoglobin 80%; color index 0.9 +.

June 15.—Antianthrax serum (30 c.c.) administered. Swelling disappearing. Pustules had a tendency to dry and form a hard crust. Patient feeling well.

Temperature: 7:30 a. m. 97.4, pulse 68; 12:00 m. 97.4, pulse 62; 4:00 p. m. 97.4, pulse 62.

Blood count: white cells 11,000; large mononuclears 9%; small mononuclears 15%; polymorphonuclears 69%; eosinophils 7%; red cells 4,080,000; hemoglobin 80%; color index 1.

June 16.—Blood count and temperature showed some disturbance, which was accounted for by the fact that, the lesions being completely sealed by crusts, the contents could not be discharged on the surface, but drained into the dependent subcutaneous tissues.

Temperature: 7 a. m. 98.6, pulse 70; 12 m. 98.6, pulse 70; 4 p. m. 98.6, pulse 70.

Blood count: white cells 16,200; large mononuclears 5%; small mononuclears 5%; polymorphonuclears 90%; red cells 4,060,000; hemoglobin 80%; color index 1.

June 17.—Scabs were raised to allow secretions beneath to drain. Smears and cultures from secretions showed no infection present.

Temperature: 7 a. m. 98, pulse 70; 12 m. 98, pulse 72; 4 p. m. 97.6, pulse 72.

Blood count: white cells 11,600; large mononuclears 19%; small mononuclears 17%; polymorphonuclears 55%; eosinophils 9%.

June 18.—Patient discharged from hospital. Urticaria developed due to the action of the horse serum. This disappeared within 24 hours after the administration of a dose of leukocytic extract subcutaneously, and the administration of bicarbonate of soda by mouth.

Temperature: 7 a. m. 98, pulse 70; 12 m. 98.6, pulse 72.

Blood count: white cells 10,600; large mononuclears 21%; small mononuclears 13%; polymorphonuclears 62%; eosinophils 4%.

The pustules from this time on were treated as simple wounds, and the patient made an uneventful recovery.

#### DISCUSSION

This case is remarkable in that there were in all 5 pustules on the patient's arm, and excision, which most authorities believe to be the classical method of treatment, was not resorted to. Antianthrax serum, it appears, played an important rôle in bringing about recovery. Following the first dose of serum all the lesions became less progressive in appearance, and the progress of the fifth pustule, which appeared on the day treatment was commenced, was arrested immediately. Furthermore, the temperature curve seems to have been influenced by something other than the patient's own reactive powers; it gradually ascended until it reached the maximum of 103 F. 36 hours after the use of the antianthrax serum had been commenced, and then decreased as immunity and convalescence became established, reaching normal in about the 84th hour.

Another interesting point is that on the 7th day after the serum treatment had been commenced, smears and cultures from secretions underneath the crusts, which were raised to permit drainage, failed to reveal the presence of the anthrax bacillus or of any other infection.

Coincident with improvement in the patient's condition the eosinophils became a factor in the blood picture. On June 13 and 14 the percentage of eosinophils increased; on June 15, when the blood count, temperature, and clinical manifestations showed signs of relapse due to drainage of secretions from the pustules into the neighboring subcutaneous tissue, the eosinophilia disappeared. When this situation was relieved, the eosinophilia reappeared and then gradually subsided as convalescence progressed. Just what the significance of the eosinophilia is we, of course, are at a loss to explain, but we deem the fact worthy of special mention.

#### EXPLANATION OF PLATE 17

Malignant anthrax pustules 11 days after infection had occurred, and 8 days after the first lesions had appeared. The photograph in natural colors was taken by Dr. David Hadden.

PLATE 17



Figure 1



Figure 2



# COMPARISON OF THE RATE OF MULTIPLICATION OF BACTERIA IN RAW MILK WITH THE RATE IN PASTEURIZED MILK \*

P. W. ALLEN

*From the Dairy Bacteriological Laboratory of the University of Illinois, Urbana*

The object of the experimentation reported here was simply to compare the rate of increase of bacteria in raw milk with that in pasteurized milk. It is realized that only an investigation of wide scope could give any satisfactory information concerning the cause of certain divergencies in the rates of increase. It is possible, however, to gain some idea of the nature and the persistency of the variations from a series of comparative counts.

Aseptically drawn milk was obtained and 200-c.c. portions were measured out. One portion was pasteurized and the other was not. After pasteurization the treatment of the two samples was the same, the object being to eliminate every factor which might cause variation.

## METHOD

Cows were selected which had a rather low udder content. At the morning's milking a quart of milk was aseptically drawn into a sterile bottle and immediately taken to the laboratory, where, after thorough shaking, 200 c.c. were placed in a sterile bottle and called Sample A. Another 200 c.c. were placed in another sterile bottle and called Sample B. The rest of the milk was labeled Sample C.

Sample A was then placed in ice water, Sample B was held for 30 minutes at 145 F., and Sample C was plated on 1% lactose agar in order to determine the number of bacteria in the milk as it came from the udder. After Sample B had been held for 30 minutes at 145 F., it was cooled to exactly 20 C. and at the same time Sample A was brought to 20 C., great care being taken that the bottles and the milk came to a constant 20 C. This was accomplished by the use of sterile thermometers and a water bath. To Sample A and to Sample B were then added the same number of bacteria from the same culture. After inoculation, Samples A and B were plated on 1% lactose agar at 2-hour intervals during 16 hours, 3 dilutions being used and 3 plates of each dilution, giving 9 plates from which to arrive at an average count of the number of bacteria in each sample at the end of each 2-hour period. The plates were incubated at 20 C. for 5 days and at 37 C. for 2 days and then counted.

\* Received for publication July 31, 1916.

The organism added to the parallel samples of milk in Series 4, 5, and 6 was a typical *Bacillus acidi-lactici* isolated from Ericsson's butter culture. It had been propagated in the milk of cow 111R.F. for 3 days previous to inoculation into the samples in which the rates of growth were to be compared, the object being to eliminate, if possible, any preliminary decrease in numbers after inoculation, due to changed environment.

The method of determining how much of the milk culture to add to the samples in order to start them off with a definite number of organisms was as follows. Careful Breed counts were made of the milk

TABLE 1  
DATA CONCERNING THE PLATING SERIES

Plating series .....	4	5	6
Date of sample of milk and of plating.....	12/20/15	12/20/15	3/22/15
Cow .....	111 L. F.	111 L. F.	191 R. F.
Amount of milk in sample, c.c. ....	200	200	200
Number of organisms in milk as it came from the udder .....	346	346	750
Organisms used for inoculation.....	<i>B. acidi-lactici</i>	<i>B. acidi-lactici</i>	<i>B. acidi-lactici</i>
Source of organism used.....	Ericsson's butter culture	Ericsson's butter culture	Ericsson's butter culture
Initial inoculation of raw sample.....	10,219 per c.c.	52,766 per c.c.	6850 per c.c.
Initial inoculation of pasteurized sample.....	10,028 per c.c.	47,366 per c.c.	7850 per c.c.
Incubation temperature, C. ....	20	20	20

culture, and from these counts it was calculated how many cubic centimeters of culture it was necessary to add to each sample in order to obtain the desired initial contamination.

In these three series the number of bacteria in the milk as it came from the udder was determined but not considered in the results of the platings, because the number of bacteria added to each sample was so large as compared with the number of udder organisms that it was judged the latter would have no appreciable effect on the results. And further it was observed that udder micrococci never appeared in

the Breed counts made for the purpose of maintaining proper dilutions in plating.

Into each of Series 8, 9, 10, 11, and 12 a chromogenic organism was inoculated, which because of the extreme color of its colony on agar could be easily distinguished on plates from udder organisms. By use of the Barber pipet<sup>1</sup> one bacterium from the selected chromogenic growth was added to a sample of raw milk and a second bacterium from the same growth was added to a sample of pasteurized milk. The bacterium had been taken with the pipet from an extremely dilute water suspension of a milk culture. In each case it had been accus-

TABLE 1—*Continued*  
DATA CONCERNING THE PLATING SERIES

7	8	9	10	11	12
5/15/16	5/30/16	6/9/16	6/17/16	6/26/16	6/26/16
189 R. F.	150 R. F.	172 R. F.	213 R. F.	111 L. F.	209 R. F.
100	100	100	100	100	100
717	240	935	163	988	1191
Bacterium 222.233 2923 Pink	Bacterium 212.233 2324 Blue	Micrococcus 222.333 3623 Orange	Bacterium 222.233 2923 Pink	Bacterium 212.232 3532 Yellow	Bacterium 212.333 2612 Orange
Barn air	Utensils	Cow's mouth	Barn air	Utensils	Utensils
115 per 100 c.c.	1 per 100 c.c.	1 per 100 c.c.	1 per 100 c.c.	1 per 100 c.c.	1 per 100 c.c.
130 per 100 c.c.	1 per 100 c.c.	1 per 100 c.c.	1 per 100 c.c.	1 per 100 c.c.	1 per 100 c.c.
20	20	20	20	20	20

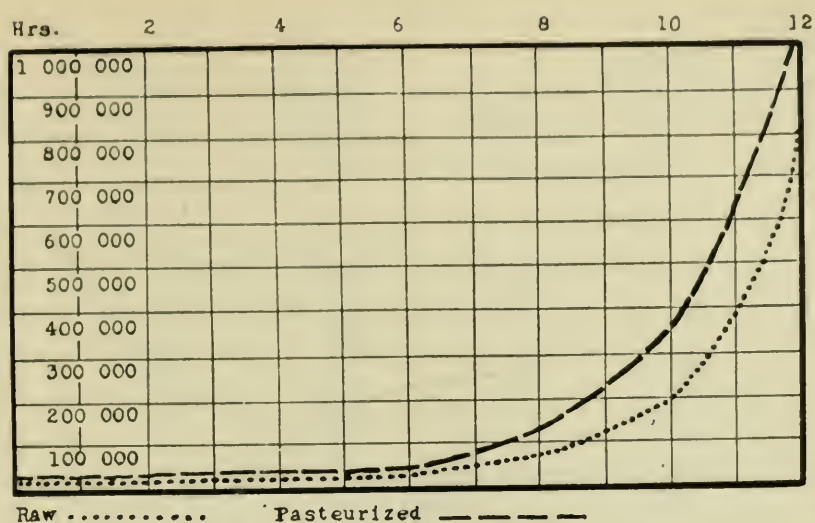
tomed to growth in the milk of the special cow used in the experiment. After the plates made from the samples of milk inoculated with these chromogenic organisms had been incubated, it was easy to pick out the special chromogenic colonies and to follow the multiplication of the single chromogenic organism placed in the sample without confusing it with udder organisms which multiplied along with it.

Graphs have not been made of Series 11 and 12 in the plating because of their similarity to the other series.

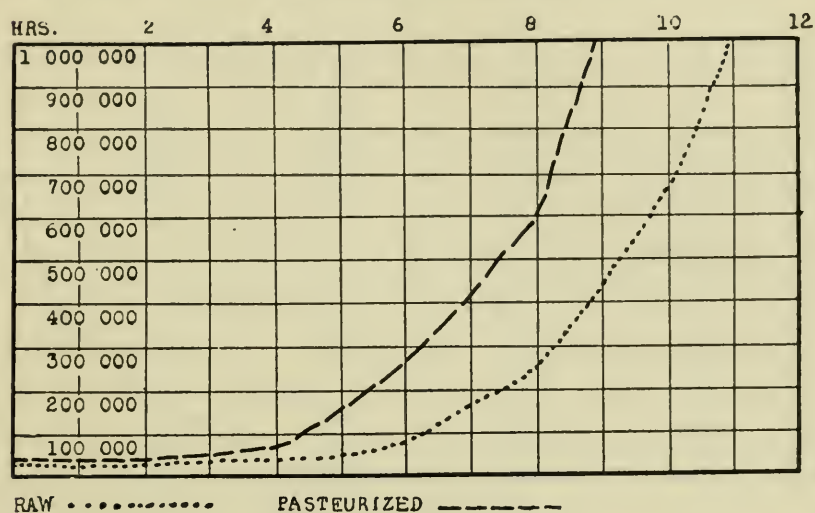
<sup>1</sup> Sc. Bull. Kansas Univ., No. 4, 1907.

TABLE 2  
COMPARISON OF THE RATE OF GROWTH OF B. ACIDI-LACTICI IN RAW MILK WITH THE RATE IN  
PASTEURIZED MILK

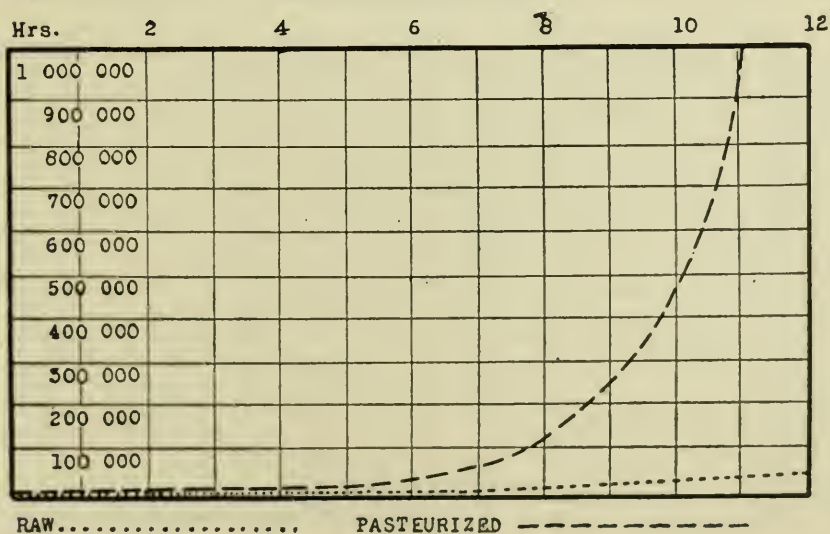
Hours After Inoculation	Average Number of Bacteria per c.c. in Raw Milk	Average Number of Bacteria per c.c. in Pasteurized Milk
SERIES 4		
0	10,219	10,028
2	11,716	13,949
4	10,083	15,616
6	29,066	38,783
8	69,366	136,999
10	183,833	369,666
12	813,833	1,065,110
14	3,935,000	5,181,666
16	8,176,666	16,515,666
SERIES 5		
0	52,766	47,366
2	47,283	46,066
4	41,749	63,483
6	80,800	247,833
8	247,999	597,166
10	672,499	1,711,666
12	1,671,666	3,121,666
14	9,293,333	14,263,333
16	16,566,666	34,216,666
SERIES 6		
0	6,850	7,850
2	11,920	16,900
4	14,600	22,300
6	15,633	41,150
8	17,533	114,650
10	33,300	467,500
12	43,000	1,565,000
14	651,600	9,599,950
16	9,683,000	12,983,300
18	33,733,300	48,566,650
20	78,100,000	101,733,000
22	162,999,000	266,000,000



Series 4

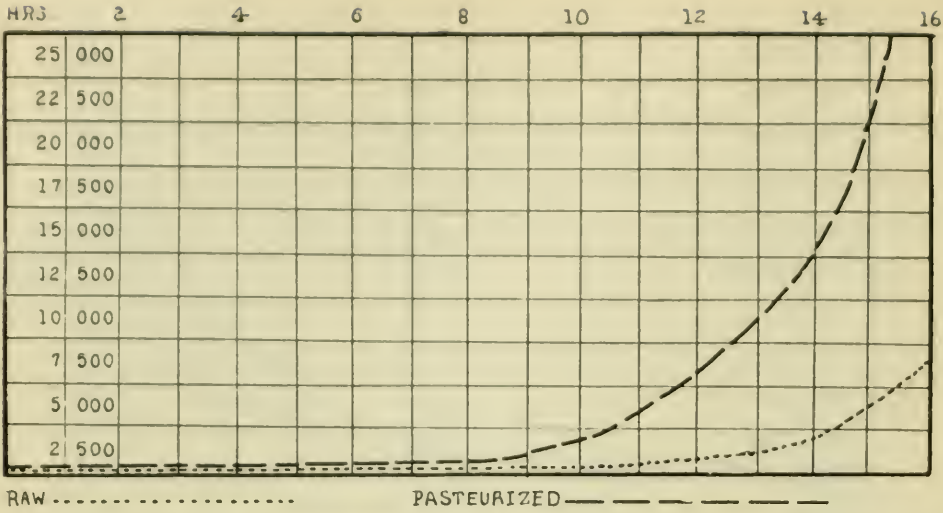


Series 5

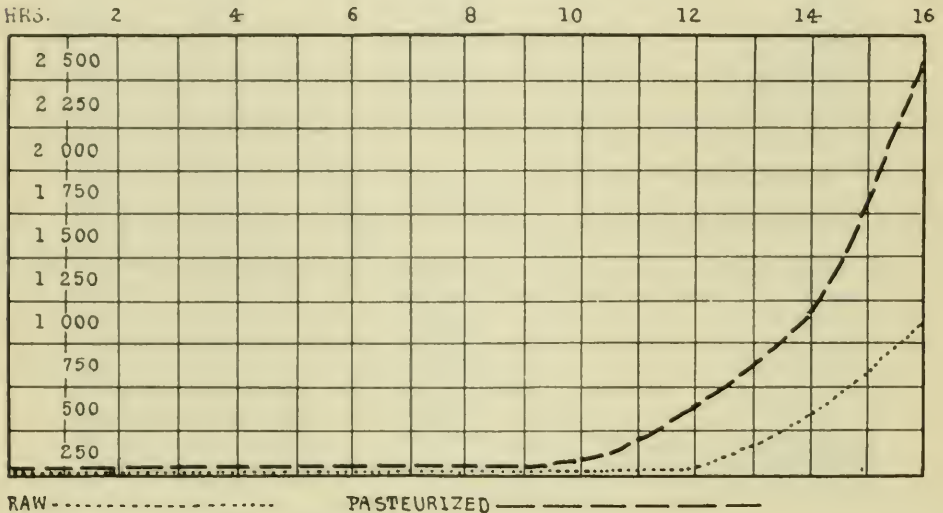


Series 6

Series 7



Series 8



Series 9

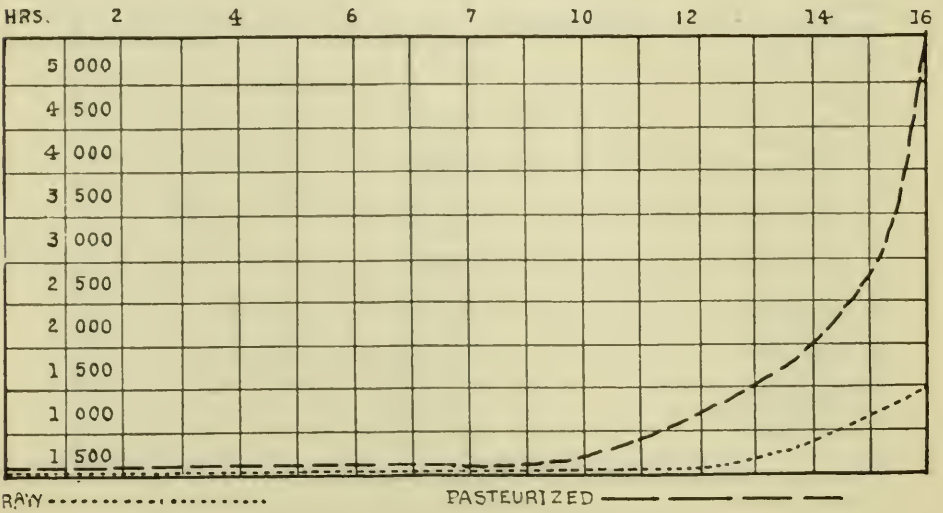
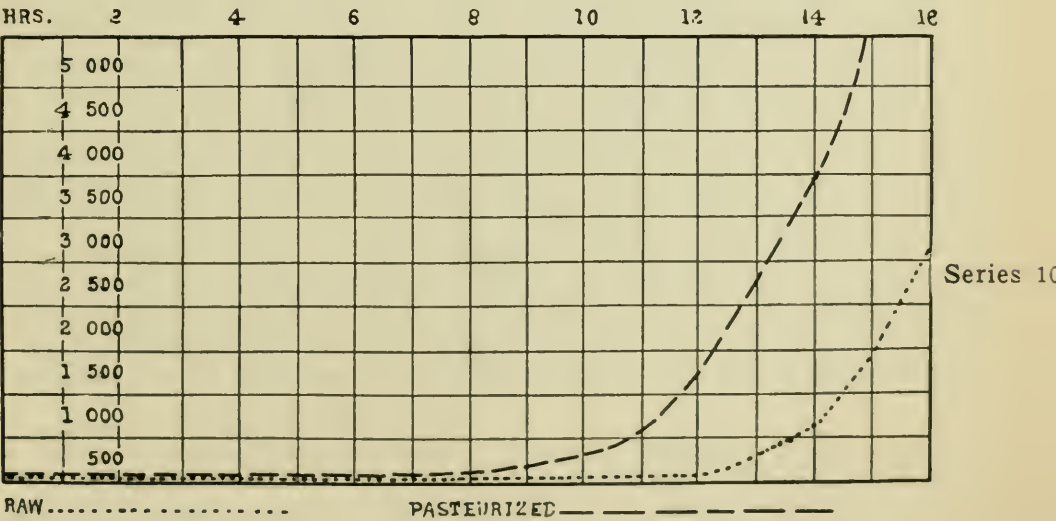


TABLE 3  
COMPARISON OF RATE OF GROWTH OF CHROMOGENIC BACTERIA IN RAW MILK WITH THE RATE  
IN PASTEURIZED MILK

Hours after Inoculation	Average Number of Bacteria per c.c. in Raw Milk	Average Number of Bacteria per c.c. in Pasteurized Milk	Hours after Inoculation	Average Number of Bacteria per c.c. in Raw Milk	Average Number of Bacteria per c.c. in Pasteurized Milk
SERIES 7			SERIES 10		
0	1.15	1.3	0	0	0
2	13	18	2	0	0
4	77	97	4	0	0
6	149	353	6	2	11
8	173	536	8	14	80
10	295	1929	10	45	326
12	778	6083	12	165	1215
14	1687	15,666	14	669	3461
16	6566	32,574	16	2738	7003
SERIES 8			SERIES 11		
0	0	0	0	0	0
2	0	0	2	0	0
4	0	1	4	0	1
6	4	10	6	7	3
8	12	27	8	15	13
10	23	63	10	33	94
12	150	368	12	100	536
14	334	900	14	418	1820
16	872	2508	16	1120	3558
SERIES 9			SERIES 12		
0	0	0	0	0	0
2	0	0	2	0	0
4	0	0	4	0	0
6	1	6	6	0	9
8	9	28	8	8	25
10	21	104	10	30	206
12	78	813	12	181	862
14	357	1469	14	615	1756
16	2979	5167	16	1658	6258



## CONCLUSIONS

Raw milk as compared with pasteurized milk exerts a powerful suppressing influence on the multiplication of certain bacteria.

When *Bacillus lactici-acidi* is accustomed to the milk of a certain cow, apparently no killing off of this organism takes place in freshly drawn milk.

When a single cell of certain pronouncedly chromogenic kinds of bacteria is added to fresh milk, the organism is found plentifully in the milk after 16 hours at 20 C., the injurious action of freshly drawn milk not being sufficiently intense to kill the one bacterial cell.

After pasteurization the organisms which remain in the milk and those which are able to get into the milk find conditions more favorable for their rapid multiplication than before pasteurization.

## EXPERIMENTAL STUDIES WITH ENDAMOEBA GROS \*

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It is but a short time since, that investigators<sup>1</sup> announced the cause of pyorrhea alveolaris as an entameba found in the pus around the root of the affected tooth. This assertion was unaccompanied by any experimental evidence. To conclude that an entameba found in the pus around the root of a tooth is responsible for the production of pyorrhea alveolaris is no more rational than to conclude that any other of the many different species of bacteria found there is responsible.

The technic pursued in the experimental study of Endamoeba Gros reported here, was as follows:

The cheesy debris covering the neck of the affected tooth is removed with a pledget of cotton held by thumb forceps. Immediately the platinum loop is sterilized in a gas or alcohol flame, and after cooling is carefully passed to the bottom of the root socket. Care must be exercised in passing the loop into the root socket for the reason that there is a marked hypervascularization of the tissues in the immediate vicinity of the root of the tooth; slight trauma causes a hemorrhage which at times is difficult to control, and the loop, which should contain a sample of pus, contains erythrocytes instead. The loop having been passed without accident into the deepest portion of the root socket of the diseased tooth, is moved upward, downward, and laterally, the object being to collect a specimen from the entire area of disease. Then the loop is carefully withdrawn from the root socket, and its content mixed with a drop of sterile salt solution on a clean slide. A cover slip is placed over the drop of emulsion and firmly pressed against the slide. The specimen is now ready for microscopic examination. Having determined the presence of active entamebas, we are ready to commence the actual isolation of the protozoa.

The technic for the preparation of the cell, and of the cover slip, the making of the capillary pipets, the centering of the pipets, and the operation of the pipet holder has been described.<sup>2</sup> The preparation of cell and cover slip having been completed, a coarse capillary pipet is made, to the free end of which is fastened a piece of rubber tubing. The free end of the tubing is placed between the lips, and the point of the pipet is immersed in sterile broth in a test tube; suction by the mouth then draws any desired quantity of broth into the pipet. With the pipet charged and carefully passed under the cover slip, a series of small droplets is deposited on the under surface of the cover slip by making slight pressure with the mouth on the rubber hose. On the completion of this step another specimen of pus is collected from around

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<sup>1</sup> Barrett and Smith: *Dent. Cosmos*, 1914, 56, p. 948. Bass and Johns: *Jour. Am. Med. Assn.*, 1915, 64, p. 554.

<sup>2</sup> Hecker: *Jour. Infect. Dis.*, 1916, 19, p. 306.

the root of the tooth as before. An emulsion of this specimen is made in the small droplets farthest from the outer edge of the cover slip. The cell is now made secure in the mechanical stage. Fine capillary pipets, to the free ends of which are fastened pieces of rubber tubing, are then charged with sterile broth and centered.

The droplets are now searched for active entamebas, and as soon as one is found, Pipet 1 is gradually moved upward until its point is faintly in focus. The cell is then moved by the mechanical stage until the protozoan is directly over the point of the pipet. Then the latter is moved upward until it comes in contact with the entameba, and the instant that it does the protozoan commences to enter. If at this time gentle suction is made with the mouth, the entameba enters the pipet very quickly. The instant that it enters the pipet, the pipet is immediately moved out of focus. We have now picked up the protozoan, but it is not yet free from all other bacteria. After the entameba has been isolated, the cell is moved to the left until a droplet of the water of condensation is found on the surface of the cover slip. The pipet is then moved upward until the point appears in this droplet. Gentle pressure is made with the mouth until the protozoan appears in the droplet and then is instantly stopped, the pipet being at the same time moved out of focus. This procedure is repeated until one has isolated the desired number of entamebas. The pipets used for this step are now removed from the pipet holder to be replaced with new pipets, which are smaller in caliber; the rubber tubes are attached to them, and they are charged with sterile broth and centered.

One of the small droplets containing an entameba and bacteria is now located. Pipet 1 is moved upward until the point is faintly in focus. The bacteria in the droplet are then collected in the pipet. As soon as all have been removed, the pipet is moved downward out of focus. As the droplet containing the entameba and the bacteria is, many times, markedly depleted in its liquor content by the removal of the bacteria, the size of the droplet is restored by moving Pipet 2 upward until the point of the pipet comes in contact with the droplet and making pressure with the mouth on the rubber hose until the desired quantity of liquor has been added. Each of the droplets containing an isolated entameba and bacteria is subjected to the foregoing technic.

The bacteria having been removed from the droplets containing the isolated entameba, the next step is that of removing the entamebas from the droplets with Pipet 2 and placing each of them in a new droplet. Then, after all the isolated entamebas have been transferred with Pipet 2 to new droplets, the pipets are removed from the holder. Two new pipets are now made, which are much larger in caliber than the ones employed for the isolation of the bacteria; the rubber hose is attached to them, and they are charged with sterile broth, and centered.

Each of the isolated protozoa is picked up with Pipet 1 and washed from 3 to 5 times in the sterile broth contained in the pipet. This rids their surfaces of any debris or bacteria which would make the experiment faulty. After each entameba has been washed, it is picked up with Pipet 2 and placed in a drop along the line on the cover slip which is at an angle to the two lines at right angles to each other.

Again the pipets are removed from the holder and one of large caliber is made, and the rubber hose attached to it. It is then charged with sterile broth, and a line is made with india ink on the shank of the pipet as a guide to the quantity of liquor it contains; it is then centered. The drop is now sought which contains the washed isolated entamebas, and they are collected

in the pipet one at a time. As soon as all of them have been collected, the pipet is moved downward out of focus. The preparation of the animal for inoculation is then commenced.

Guinea-pigs of standard size are used. The animal, wrapped in a large towel, its head exposed, is placed on its back in the lap and grasped gently by the thighs to prevent its moving. The pipet is removed from the holder and the shank of the pipet grasped by the thumb and index and second fingers of the left hand. With the left hand the rubber hose is placed between the lips. Traction is then made on the lip of the guinea-pig until the gingiva is freely exposed, whereupon the point of the pipet is carefully passed between the gum and the root of the tooth until the deepest portion of the gingiva is reached. The instant that this point is reached gentle pressure is made with the mouth on the free end of the rubber hose held by the lips, the liquor in the pipet gradually passes into the gingival space, and as soon as it reaches the guide line on the shank of the pipet, the pressure is stopped and the pipet immediately withdrawn. The guinea-pig is laid aside for 1 hour. At the end of this time it is unwrapped and placed in its pen, the date, the point of inoculation, and the dose having been noted.

The gingival space of the left and right upper and lower central incisors, meso-approximal and distal surfaces, were thus injected in 6 guinea-pigs. The number of organisms inoculated ranged from 5 to 18. Careful macroscopic and microscopic examinations at the point of inoculation were made daily, but, with the exception of 3 guinea-pigs which showed slight inflammation of the gingivae on the 2nd and 3rd days respectively, none of the animals disclosed inflammation, pus, or presence of entamebas during a period of 84 days following the inoculation.

The author also injected active entamebas into the gingival space around the teeth in his own mouth. Careful microscopic examination was made of the contents of the gingival space of each of the 10 teeth selected for inoculation, and in no instance did the author find entamebas. The teeth selected were the 6 lower anterior teeth and the 4 anterior upper teeth.

The entamebas were isolated from an emulsion of pus from around the root of the affected tooth in the same manner as described in connection with the animal experiments. The difficult step was the development of a technic for the inoculation of the gingival space by the author himself, unassisted. The following method became easy after a little practice.

The pipet employed is the same in type as that used in the animal experiments. The entameba having been picked up, the pipet is lowered out of focus. The gum overlying the site selected for inoculation is then carefully massaged, and the neck of the tooth is wiped to free the gingival margin of all debris. The pipet is then removed from the pipet holder, and the shank grasped by

the thumb and index finger of the right hand, while the left holds the rubber bulb of an ear syringe, which is attached to the free end of the rubber tubing attached to the pipet. The worker, seated in front of a mirror, gently passes the point of the pipet into the deepest portion of the gingival space, until the point meets with resistance. Gentle pressure with the left hand on the bulb of the ear syringe forces the liquor slowly from the pipet into the gingival space; as soon as the liquor reaches the guide line on the shank of the pipet, the pressure is stopped and the pipet is instantly withdrawn. The inoculated area is given a liberal coating of the compound tincture of benzoin, which is allowed to harden. The traction on the lip is stopped, and the lip allowed to return to its normal position.

The largest number of active entamebas thus injected into the gingival space was 14. Macroscopic examinations were made of the area of inoculation daily for 7 days following. After the 7th day microscopic examinations were made. The first experiment was commenced on March 12, 1916, and the succeeding experiments followed at 5-day intervals. The areas of inoculation were carefully observed daily until August 1, 1916, and up to this time no inflammation of the gum had occurred, and no pus, and no entamebas had been found microscopically.

From the foregoing experiments we learn that the gingival space of the guinea-pig and the healthy gingiva of man, when inoculated with active entamebas, do not develop inflammation or pus and that the entamebas do not survive to reproduce their species. Hence, at this time, we can not accept the theory that this protozoan, when found in the pus around the root of a tooth affected by pyorrhea alveolaris, is etiologically responsible for the production of the malady.

# CULTURE MEDIA FOR GONOCOCCUS \*

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## HISTORICAL REVIEW

In 1879 Neisser<sup>1</sup> discovered Gonococcus. Bumm<sup>2</sup> (1885) was the first of many investigators to obtain satisfactory results in its cultivation. He used human placental blood serum as the medium. Wertheim<sup>3</sup> (1891) obtained a luxuriant growth using serum agar. As substitutes for human serum, several protein-containing fluids and animal serum have been tried by many workers; for example, hydrocele fluid by Steinshneider<sup>4</sup> (1890), ovarian, cystic, and hydrosalpinx fluid by Menge<sup>5</sup> (1893), and ascites and hydrothorax fluid by Kiefer<sup>6</sup> (1895). Scholtz<sup>7</sup> noticed that the amount of protein in these fluids varied, so that the gonococcal growth was not constant, while Laitiner reported that the alkalinity of cystic and ascitic fluid has a great influence on the growth. J. Koch<sup>8</sup> recorded fair results with ascitic agar, and Wassermann<sup>9</sup> with pig serum nutrose agar. Stross,<sup>9</sup> in a comparative study of human and animal sera, found that the gonococcus grew readily on human serum but not on animal serum. Schrötler and Winkler<sup>10</sup> (1890) used peewit protein with satisfactory results. Preparations of proteins of other kinds were used by Finger, Ghon, and Schlagenhauser,<sup>11</sup> protein-containing urine agar by De Christmas,<sup>12</sup> egg yoke by Steinschneider,<sup>13</sup> and egg-white agar by Lipschütz,<sup>14</sup> but none of these media was satisfactory in the hands of other workers. Abel<sup>15</sup> introduced Peiffer's blood agar, and later J. Koch used horse-blood agar with excellent results. M. Seé<sup>16</sup> found rabbit-blood agar unsatisfactory. Thalman<sup>17</sup> was the first to secure growth of the gonococcus on ordinary culture medium; he pointed out that plain agar must be exactly neutralized. Later his work was corroborated by Brongersma and Van de Velde,<sup>18</sup> Picker,<sup>19</sup> and others, but Baermann,<sup>20</sup> Rotmann,<sup>21</sup>

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<sup>1</sup> Centralbl. f. d. med. Wissensch., 1879, 17, p. 497.

<sup>2</sup> Deutsch. med. Wchnschr., 1885, 11, p. 508.

<sup>3</sup> Ibid., 1891, 17, p. 1351.

<sup>4</sup> Berlin klin. Wchnschr., 1897, 34, p. 379.

<sup>5</sup> Zentralbl. f. Gynäk., 1893, 17, p. 153.

<sup>6</sup> Berlin klin. Wchnschr., 1895, 32, p. 332.

<sup>7</sup> Arch. f. Dermat. u. Syph., 1899, 49, p. 3.

<sup>8</sup> Kolle and Wassermann's Handb. d. pathogen. Mikroorganismen, 1913, 4, p. 655.

<sup>9</sup> Berlin klin. Wchnschr., 1897, 34, p. 685. Ztschr. f. Hyg. u. Infektionskrankh., 1898, 27, p. 298.

<sup>10</sup> Centralbl. f. Bakteriöl., I, O., 1905, 38, p. 491.

<sup>11</sup> Arch. f. Dermat. u. Syph., 1894, 28, p. 3.

<sup>12</sup> Ann. de l'Inst. Pasteur, 1897, 9, p. 609; 1900, 14, p. 331.

<sup>13</sup> Berlin klin. Wchnschr., 1895, 32, p. 984.

<sup>14</sup> Centralbl. f. Bakteriöl., I, O., 1904, 36, p. 743.

<sup>15</sup> Deutsch. med. Wchnschr., 1893, 19, p. 265.

<sup>16</sup> Ann. de dermat. et de syph., 1900, 31, p. 889.

<sup>17</sup> Centralbl. f. Bakteriöl., I, O., 1900, 27, p. 828; 1902, 31, p. 678.

<sup>18</sup> Centralbl. f. Bakteriöl., I, O., 1903, 33, p. 311.

<sup>19</sup> Wien. klin. Wchnschr., 1906, 19, p. 122.

<sup>20</sup> Ztsch. f. Hyg. u. Infektionskrankh., 1904, 43, p. 529.

<sup>21</sup> Monatsh. f. prakt. Dermat., 1905, 41, p. 516.

Alfvén,<sup>22</sup> and Jackstadt<sup>23</sup> did not obtain the same results. Urbahn,<sup>24</sup> Wildbolz,<sup>25</sup> Vannod,<sup>26</sup> Martin,<sup>27</sup> and others, succeeded in getting a growth on ordinary agar. Of these, Vannod noted that slight alkalinity of the medium was necessary. Recently Alcock reported a luxuriant growth of the gonococcus on ordinary peptone agar. In 1913 Sabouraoud and Noire,<sup>28</sup> and later Weil and Noire,<sup>29</sup> used whey agar successfully. In the same year Besredka and Jupille<sup>30</sup> found egg broth a suitable medium.

According to the investigations cited, we have no really satisfactory media for the cultivation of the gonococcus. I have hitherto used horse- or goat-blood agar with satisfactory results. Recently, however, I have prepared and used, as a substitute for blood agar, the medium to be described.

#### THE PREPARATION OF THE MEDIUM

Two hundred cubic centimeters of cow's milk are warmed to 60 C. and 5% ascitic agar is added drop by drop, the milk being shaken to cause precipitation of the casein. It is then filtered through filter paper. To the filtrate is added 10% caustic-soda solution up to the point of slight alkalinity, and then 2 gm. of urea. The clear colorless fluid is sterilized at 60 C. for 30 minutes every day for 3 days. The sterilized fluid is then mixed with melted 0.3% nutrose agar at 45 C. in the proportion of 1 part of the fluid to 2 parts of agar. Plate or slant forms are prepared as needed. The medium must be incubated before use to make sure that no micro-organisms have gained access during preparation. When fluid media are needed, equal parts of the fluid and of ordinary broth or peptone water are mixed.

It is very necessary that the fluid should not be heated above 70 C., as unsatisfactory results frequently follow the use of the fluid heated at a higher temperature. For the precipitation of casein the amount of acid is not constant, the different samples of milk undoubtedly varying in quality.

#### APPEARANCE OF THE GONOCOCCUS IN CULTURE

Six strains were cultivated, 4 of which grew well after 48 hours at 37 C. and 2 after 5 days. The results with the four strains may be outlined as follows:

1. Whey agar at 37 C. After 24 hours the gonococcus colony was macroscopically hardly visible, round, about 1 mm. in diameter—a slightly elevated disc with moist-looking surface, semitransparent, of smooth margin, and of light grayish-blue color. After 48 hours, the colony, macroscopically, was small, round, about 2 mm. in diameter—

<sup>22</sup> Hygiea, 1904, 66, p. 151.

<sup>23</sup> Dissertation, Königsberg, 1904.

<sup>24</sup> München. med. Wehnschr., 1903, 50, p. 1529.

<sup>25</sup> Centralbl. f. Bakteriöl., I, O., 1902, 31, p. 128.

<sup>26</sup> Ibid., 1905, 44, pp. 10, 110.

<sup>27</sup> Jour. Path. and Bacteriol., 1910, 15, p. 76.

<sup>28</sup> Ann. de dermat. et de syph., 1913, 4, p. 439.

<sup>29</sup> Compt. rend. de Soc. de biol., 1913, 74, p. 1321.

<sup>30</sup> Ann. de l'Inst. Pasteur, 1913, 27, p. 1009.

an elevated disc of viscous consistency. When touched with a platinum loop the colonies were readily removed from the medium. Surface moist and shining. Semitransparent. Margin smooth. Color grayish-blue. Centers of colonies light-brown. Microscopically the colony was a finely granular, elevated disc with uniform margin, and of a faint yellowish-dark color. Center of colony humpy and opaque. After 72 hours, macroscopically, the colony was still small and round—an elevated disc of sticky consistency, harder to remove from the medium. Margin somewhat dried and undulated. Color gray. Center opaque. Microscopically it was granular and a little coarser, with iridescent surface and wavy margin. Yellow-dark in color. Center opaque. After 96 hours, 120 hours, 5 days, and 6 days the appearance of the colony was unchanged except that after 120 hours the surface was considerably enlarged and flat.

2. Whey broth. After from 24 to 48 hours the fluid of all strains was clear and colorless, but finely granular grayish-white sediments appeared. After from 4 to 6 days the fluid was still clear and the colonies appeared as fine crummy particles, which readily sank to the bottom when lightly shaken.

3. Whey peptone water. In this medium the growth was not so good as that in the whey broth just described.

4. Whey sugar litmus medium. A growth similar to that in whey peptone water.

#### CONCLUSIONS

Blood agar and blood broth are still the most suitable culture media for growth of the gonococcus. If blood cannot easily be obtained, however, whey agar or whey broth can be used as substitutes for blood agar and blood broth.



# THE EFFECT OF TOLUENE ON THE PRODUCTION OF ANTIBODIES \*

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In connection with experiments on the effects of benzene on the production of antibodies, experiments of a similar nature were made with toluene. The preparation used was "Toluene Merck-Pure." Practically the same methods were followed as in the work on benzene, and the titers of the antibodies studied were determined as described in the report of that work.<sup>1</sup>

The first experiment was designed to test the action of toluene injected subcutaneously on the 4th day after an intraperitoneal injection of 30 c.c. of sheep blood, and daily for 8 days thereafter. The 3 rabbits used and the control were of the same litter, and weighed about 1500 gm. each. The daily quantity of toluene in each case was

TABLE 1

THE FORMATION OF LYSIN AND PRECIPITIN IN RABBITS INJECTED WITH TOLUENE DAILY FOR 8 DAYS, BEGINNING WITH THE 4TH DAY AFTER THE INJECTION OF SHEEP BLOOD

Days After Injection of Sheep Blood	Injections of Toluene	Rabbit 1		Rabbit 2		Rabbit 3		Control (no toluene)	
		Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin
2		768	0	384	0	768	0	768	0
4		12288	0	3072	0	384	0	768	0
5	+								
6	+	24576	180	24576	180	12288	180	6144	50
7	+								
8	+	12288	1800	12288	1800	6144	200	6144	800
9	+	12288	1800	12288	1800	6144	50	6144	1600
10	+								
11	+	12288	1800	12288	1800	6144	80	12288	3200
12	+	6144	1800	3072	1800	1536	50	12288	6400
13		6144	1800	6144	1800	1536	40	6144	12800
15		Died		6144	1800	3072	25	3072	12800
16				6144	1800	3072	0	3072	12800
17				6144	1800	1536		3072	12800
20				6144	1800	768		3072	12800
23				6144	1800	768		3072	12800
28				1536	800	768		3072	12800
36				3072	400	768		6144	3200
42				3072	50	768		1536	1600
49				1536		768		1536	800
58				192		48		96	200

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<sup>1</sup> Jour. Infect. Dis., 1916, 19, p. 69.

TABLE 2

THE EFFECT ON THE PRODUCTION OF LYSIN AND PRECIPITIN OF 12 DAILY INJECTIONS OF 1 C.C. OF TOLUENE PER KILO OF WEIGHT, THE FIRST ON THE SAME DAY THAT 30 C.C. OF SHEEP BLOOD WERE INJECTED INTRAPERITONEALLY

Days After Injection of Sheep Blood	1		2		3	
	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin
1	96		192		24	
2	95		384		24	
3	192		768		24	
4	1536	10	1536	10	768	
5	24576	100	12288	100	6144	25
6	24576	100	12288	100	12288	100
7	12288	600	12288	600	12288	400
8	12288	1200	12288	1200	12288	3200
9	12288	1200	12288	1200	6144	3200
10		1200	12288	1200	6144	3200
11	24576	1200	6144	2400	6144	3200
12	24576	2400	12288	2400	6144	6400
13	12288	2400	12288	4800	6144	6400
14	12288	2400	12288	4800	3072	6400
15	12288	2400	12288	2400	3072	12800
16	12288	2400	12288	2400		
17					1536	3200
18	12288	2400	12288	2400		
19					1536	3200
20	12288	2400	12288	2400		
22	12288	2400	12288	2400		
24					768	800
25	12288	2400	12288	2400		
26						
28						
30	6144	2400	3072	2400		
31					1536	800
35	6144	2400	3072	2400		
38					768	800
40	12288	2400	1536	9600		
43						
45					768	800
47	6144	6400	768	16800		
50						
52					768	400
54	12288	6400	384	6400	Discharged	
58						
61	6144	3200	384	12800		
64						
66						
68	12288	3200	384	12800		
75	6144	1280	384	10240		
82	3072	3200	384	12800		
85						
87						
89	6144	3200	384	12800		
94						
99	3072	6400	192	12800		
101	3072	6400	192	12800		
117	1536	9600	384	9600		
122	6144	2400	1536	19200		
132	6144	9600	197	160000		
148						
162	1536	9600	384	9600		
170	1536	9600	384	9600		
178	1536	9600	384	25600		
188	768	4800	384	12800		
198	1536	4800	192	7700		
205	1536	4800	192	9600		
212	1536	4800		4800		
231	768	800	768	400		
237	768	400	384	800		
243	-	Died	192	200		

Rabbits 1 and 2 received 1-1.5 c.c. benzene subcutaneously daily from the 96th to the 109th day.

TABLE 2—Continued

THE EFFECT ON THE PRODUCTION OF LYSIN AND PRECIPITIN OF 12 DAILY INJECTIONS OF 1 C.C. OF TOLUENE PER KILO OF WEIGHT, THE FIRST ON THE SAME DAY THAT 30 C.C. OF SHEEP BLOOD WERE INJECTED INTRAPERITONEALLY

4		5		Control (no toluene)		Control (no toluene)	
Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin
						584	
6144		6144		12288	400	768	25
49152	400	12288	100	12288	1600	12288	200
12288	800	12288	800			24576	1600
				49152	25600	49152	3200
6144	2400	6144	1200			49152	9600
12288	4800	12288	1200	49152	12800	49152	4800
12288	9600	12288	2400	49152	6400	24576	4800
	19200	3072	9600			24576	1600
				24576	6400	6144	3200
1536	6400	1536	3200	12288	4800		
1536	4800	3072	2400			1536	800
				6144	4800		
768	12800	384	3200	3072	4800		
384	9600	384	3200				
768	9600	768	3200	3072	4800	768	200
768	9600	384	4800	1536	400		
				1536	200		
384	19200	384	4800			768	200
384	9600	384	1200	1536	200		
				1536	160	768	200
192	4800	768	4800				
192	2400	1536	4800	1536	0	768	0
				Discharged		768	0
384	300	1536	3200			Discharged	
192	12800	384	6400				
384	800	384	600				
Discharged		384	0				
		192	0				
		96					
		Discharged					

1 c.c. in 1 c.c. of olive oil. No change in the number of leukocytes in the blood was observed and the general health of the animals remained good, tho one rabbit died after 2 weeks. The results as to the production of lysin and precipitin are given in Table 1. The principal effect of the toluene appears to have been a reduction in the amount of precipitin.

In the next experiment the injection of toluene was commenced on the same day as the injection of sheep blood. Five rabbits, weighing about 1000 gm. each, were given 30 c.c. of sheep blood intraperitoneally and 1 c.c. of toluene subcutaneously. The injections of toluene were repeated each day for 12 days. No definite change resulted in the number of leukocytes. The results as to lysin and precipitin are shown in Table 2. While there may have been some restraint in the early production of antibodies, the outstanding feature was the persistence of lysin and precipitin longer and with greater fluctuation in most of the toluene rabbits than in the controls (rabbits injected with the same amount of sheep blood, but not with toluene). That the daily bleeding of some of these rabbits in the early period of antibody-production had some influence on the output of antibodies cannot be excluded, but that the later fluctuation and persistence of the lysin and precipitin resulted wholly from the effects of the bleedings cannot be maintained, because similar deviations from the usual course occurred in experiments with toluene in which the bleedings were not so frequent; and it may be pointed out too that so far as my observations go the removal of large amounts of blood from recently immunized animals does not disturb the typical curve of the antibody course in the blood, but tends rather to increase the antibody output. Hence it probably is justifiable to ascribe the unusual fluctuation and persistence of lysin and precipitin in the toluenized rabbits to the action of toluene combined perhaps with the effects of daily bleedings during the first two weeks after the injection of antigen.

Rabbits 1 and 2, Table 2, require special consideration. It is noteworthy that in these animals there occurred an increase in the titer of the precipitin as late as 40-50 days after the injection of the antigen and a persistent but fluctuating high titer of both lysin and precipitin thereafter for a long period. From the 96th to the 109th day they received daily, at first 1 c.c., and then 1.5 c.c. of benzene subcutaneously, and, as mentioned in the article on benzene, seemingly without any immediate effects either on the leukocytes, the general

health of the animals, or on the course of the lysin and precipitin. This general result is in harmony with the results of the injection of benzene at the height of antibody-production in rabbits not previously injected with toluene.<sup>1</sup> Since in the latter animals the production of lysin and precipitin persisted longer and underwent more fluctuation than under ordinary circumstances, it is altogether reasonable to conclude that the treatment with benzene of the toluenized rabbits, 1 and 2, tended to increase still further the persistence and fluctuation of the lysin and precipitin. Certainly the persistence of these antibodies in the concentration shown in the table for some 243 days after the injection of the antigen forms a noteworthy deviation from the usual course of antibodies in general and of antishoop precipitin in particular.

Experiments designed to test the action of toluene injected for some days before the introduction of antigen may be summarized as follows: Five rabbits received daily 1 c.c. of toluene per kilo of weight for from 10 to 15 days before the injection of sheep blood. All save

TABLE 3

THE EFFECT ON ANTIBODY-PRODUCTION OF DAILY INJECTIONS OF TOLUENE, 1 C.C. PER KILO OF WEIGHT, FOR FROM 10 TO 15 DAYS BEFORE INJECTION OF SHEEP BLOOD

Days After Injection of 30 c.c. Sheep Blood	1		2		3		4		5	
	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin
1					0		0		0	
2					0		0		96	
3					768	0	768	0	1536	0
4	48	0	1536	0	12288	50	12288	50	12288	50
5					6144	100	12288	100	12288	100
7	1536	0	12288	800	24576	400	24576	400	24576	600
8					12288	400	12288	800	24576	800
9					24576	400	24576	800	24576	800
10	3072	0	12288	6400	24576	400	24576	400	24576	800
13	3072	0	12288	6400	12288	800	12288	1200	24576	1200
15					12288		6144	2400	6144	1200
16	3072	0	6144	6400						
18					6144	600	3072	600	6144	400
20	768	0	1536	3200						
22					6144	400	3072	600	6144	1200
23	1536	0	1536	12800						
27	768	0	1536	200000						
31	768	0	768	0						
34	768	0	768	0						
35			Died		3072	200	1536	400	3072	800
38	384	0								
45	192	0			768	100	536	200	3072	800
55	384	0			Died		Died		1536	400
64	384	0							Died	
69	192	0								
85	384	0								
94	384	0								
105	384	0								

one died without obvious cause before the production of antibodies had run its course. The survivor, Rabbit 1, Table 3, gave no precipitin at all, and only a small amount of lysin, probably because of an inherent lack of power to produce antibodies. Rabbit 2, Table 3, of another series, which like Rabbit 1 received toluene for 10 days, approached the normal average of lysin and precipitin, but the very high concentration of precipitin on the 27th day, followed by an apparent total loss of this antibody, is remarkable and exceptional. Rabbits 3, 4, and 5, Table 3, which received toluene for 15 days, produced about as much lysin as normal rabbits but not nearly as much precipitin. Taken altogether, these results indicate that prolonged toluene treatment antecedent to the injection of antigen may reduce the output of precipitin.

To test the effect of toluene when introduced in the course of antibody-formation, injections of toluene were commenced on the 12th day after 30 c.c. of sheep blood had been injected. Table 4 illustrates the procedure as well as the results. It appears that lysin was reduced for a few days, but it is not evident that precipitin was affected. However, it is noteworthy that the titers of lysin and precipitin remained well above normal for some 200 days, far beyond the limit in untreated rabbits.

The effects of smaller doses of toluene have also been studied but not extensively. The subcutaneous injection of 0.01 c.c. of toluene per kilo of weight each day for 12 days, beginning on the same day that sheep blood was injected intraperitoneally, had no depressive effect on the production of lysin and precipitin. The rabbits (4) all died about the 80th day after the injection of sheep blood, the lysin titers then being 1536, 768, 1536, and 192, and the corresponding precipitin titers 3200, 400, 800, and 1600. These values, especially those of the precipitin, are higher than is usually the case at the same period in rabbits which have been injected with sheep blood only.

The injection of 0.005 c.c. of toluene per kilo of weight daily for 4 days before, and 10 days after, the injection of sheep blood had no appreciable effect on antibody-production so far as could be observed, but the rabbits (3) all died within 50 days of the introduction of the blood.

This high death rate of the rabbit has been a serious handicap, especially in that it is necessary to keep the animals under observation over long periods. This is one reason why the results now presented are somewhat fragmentary and inconclusive.

TABLE 4

THE EFFECT OF TOLUENE WHEN INJECTED AT THE HEIGHT OF ANTIBODY-PRODUCTION

Days After Injection of 30 c.c. of Sheep Blood	Injections of Toluene, c.c.	Lysin	Precipitin
1		0	
2		0	
3		96	
4		3072	
5		24576	100
6		24576	600
7		12288	600
8		24576	600
10		24576	2400
11		12288	2400
12	0.3	6144	2400
13	0.3	6444	2400
15	0.5	768	4800
17	0.5	768	3200
18	0.5	768	3200
20	0.5	768	3200
22	0.75	3072	3200
25	0.75	1536	2400
28	0.75	1536	1600
32	0.75	1536	1200
35		3072	2400
42		1536	2400
50		1536	3200
59		768	2400
70		768	1200
77		1536	1200
84		1536	3200
94		768	1600
103		768	400
113		768	800
129		384	800
145		384	400
161		384	200
174		192	400
186		384	200
203		384	50
216		192	0

The results of the experiments indicate that toluene injected into rabbits as outlined in this report reduces the output of antibody in the blood, especially of precipitin, during the early stages of antibody-production; under certain circumstances, however, as when injected while the antibody content of the blood is at its height, or as shown in Table 2, toluene appears to cause greater late fluctuation and longer persistence of new antibody than occur under ordinary conditions. Even relatively small doses of toluene may have effects of this kind. As already explained, it does not seem likely that such effects, which are obtainable with benzene also, can result from repeated bleedings.

A few facts and considerations bearing on the question of how toluene produces its effects may be discussed briefly. It might be said that toluene in some way interferes with the absorption and fixation of the antigen, but so far as the results of

observations with the precipitin method go, the course of the antigen—sheep protein—in the blood appears to be quite the same in rabbits injected with toluene (and benzene<sup>1</sup>) as in rabbits not so injected. Furthermore, the fact that toluene injected as late as 12 days after the introduction of antigen appears to prolong greatly the period of persistence of antibody in the blood (Table 4), speaks against the assumption of a delay and change in the absorption and fixation of antigen.

In the course of this work I confirmed the results of Kline and Winternitz<sup>2</sup> that toluene in the quantity of 1 c.c. per kilo does not affect the number and proportion of leukocytes in the blood of rabbits, at least in the early stages after its introduction. No observations were made in the later stages. The phagocytic activity in vitro of the leukocytes of toluenized rabbits did not appear to be reduced—a circumstance which is in harmony with the observation of Kline and Winternitz that there is no difference in response to experimental pneumococcus infection between rabbits treated with toluene and normal rabbits. Likewise the observation of Kline and Winternitz that the marrow of rabbits injected with toluene (1 c.c. per kilo) presents a hyperplasia, especially of the myeloid cells, there being only slight changes in the spleen and lymph glands, is confirmed by the results obtained incidentally to the study of the effects of toluene on antibody-formation. The most marked changes seemed to develop in the marrow of the femur, the next most marked in that of the humerus, while in that of the tibia, ulna, radius, ribs, and spinal column the changes were only slight. The myelocytes played the chief rôle in this toluene hyperplasia of the marrow, the nongranular and amphophil cells being far in excess of other kinds of cells. The hyperplasia subsided quickly when the injection of toluene was stopped, so that in about 6 days the number of cells was normal again. From then on, the number of cells in the marrow of the toluenized rabbits appeared to be smaller than normal, the cells being replaced by a homogeneous substance with but few fat cells. During the period of hyperplasia the giant cells of the marrow seemed more actively phagocytic for leukocytes than under normal conditions. There were no noteworthy changes attributable to the effects of toluene in any of the other tissues of the body.

Toluene, then, has a direct effect on cells in the marrow, which, as

<sup>2</sup> Jour. Exper. Med., 1914, 18, p. 50.

developed more fully in the report on benzene, there are many reasons to believe are concerned directly with the elaboration of antibodies. As this effect is observed only for a short time after the injection of toluene, it may be the explanation of why toluenized rabbits produce less antibodies than normal rabbits in the earlier stages of antibody-formation. On the other hand, as the abnormal fluctuation and persistence of antibody in toluenized rabbits occur at a time when the cells of the marrow, if anything, are fewer in number than normally, we are left without any apparent morphologic basis for an explanation of these interesting phenomena. Is there in such animals a late increase in the production of antibodies, or are conditions established that interfere with the passage of accumulated antibodies out of the blood? This and other questions can be answered only by further investigation.

#### SUMMARY

In rabbits the effect of toluene in repeated doses of about 1 c.c. per kilo of weight is to diminish the output of antibody in the earlier periods of antibody-production, and, under certain conditions, to cause prolonged persistence of antibody in the blood, with, in at least some cases, marked fluctuations in the concentration.

Increased persistence of antibody in the blood may follow smaller doses of toluene also.

There is no immediate change as to number and proportion of leukocytes in the blood of rabbits treated with toluene as here described, nor is there any change in the phagocytic activity of the leukocytes *in vitro*.

In rabbits receiving the larger doses of toluene there occurs a transitory myeloid hyperplasia in the bone marrow, but later no marked changes in the marrow or other organs appear. On this account there is at present no basis for concluding whether the fluctuation and persistence of antibodies in the blood is better explained as resulting from increased and prolonged production of antibodies, or as resulting from interferences with the passage of antibodies out of the blood.

## ANAPHYLACTIC SHOCK IN DOGS \*

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The rapid and pronounced fall in blood pressure which is characteristic of anaphylactic shock in the dog was first observed by Richet<sup>1</sup> in his studies on congestion.

This phenomenon, which constitutes the only constant feature when the animal is anesthetized, was carefully studied by Biedl and Kraus.<sup>2</sup> They sensitized dogs by the injection subcutaneously of from 3 to 5 c.c. of horse or ox serum, and 21 days later gave 10 c.c. of the same serum intravenously. After an interval of from 10 to 15 seconds the blood pressure fell from the normal, 150 to 180 mm. of mercury, to 80 or even 40 mm. During this stage stimulation of the peripheral end of the splanchnic nerve and the intravenous injections of from 0.1 to 0.2 mg. of epinephrin caused either no rise, or only a slight rise, in blood pressure. Barium chlorid, on the other hand, by its power of vasoconstriction, raised the pressure and stopped the symptoms. From this they concluded that the fall in pressure was due to vasodilatation caused by injury to the peripheral vasomotor apparatus. They did not believe that the heart was in any way concerned in the process. In their further studies they still adhered to this view.<sup>3</sup>

This conclusion of Biedl and Kraus has been accepted by most other investigators, notably by Pearce and Eisenbrey,<sup>4</sup> Edmunds,<sup>5</sup> and others. Pearce and Eisenbrey further localized the injury in the nerve endings rather than in the muscular coat of the vessel wall. They think, however, that these structures are not completely paralyzed, but that their activity is greatly diminished as shown by the slight reaction to epinephrin and stimulation of the splanchnic nerve. The fall in blood pressure is due to the accumulation of the blood in the large venous trunks and the vessels of the splanchnic area as a result of vasodilatation, so that not enough blood reaches the heart to keep up the pressure in the systemic circulation.

Robinson and Auer,<sup>6</sup> by use of the electrocardiogram, found evidence that anaphylactic shock in the dog may produce definite cardiac disturbances in addition to the peripheral vasomotor paralysis.

W. H. Schultz,<sup>7</sup> working chiefly with cats and to a less extent with dogs, offers a quite different explanation for the fall in blood pressure in anaphylactic shock. According to his view, the injection of the second dose of serum causes

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<sup>1</sup> Compt. rend. Soc. de biol., 1905, 58, p. 112.

<sup>2</sup> Wien. klin. Wchnschr., 1909, 22, p. 363.

<sup>3</sup> Ztschr. f. Immunitätsf., 1910, 7, p. 205.

<sup>4</sup> Jour. Infect. Dis., 1910, 7, p. 565.

<sup>5</sup> Ztschr. f. Immunitätsf., 1913, 17, p. 105; 1914, 22, p. 181.

<sup>6</sup> Jour. Exper. Med., 1913, 18, p. 556.

<sup>7</sup> Jour. Pharm. and Exper. Therap., 1912-13, 3, p. 299. Bull. Hyg. Lab., P. H. and M.-H. S., No. 80, 1912.

a marked constriction of the vessels of the lungs. This is so extreme that too little blood reaches the left side of the heart to maintain the pressure in the systemic vessels. The accumulation of blood in the large venous trunks and in the splanchnic area is, according to Schultz, "for the most part purely passive." This view has apparently been accepted by Dale<sup>8</sup> and Airila;<sup>9</sup> but it has been severely criticized and apparently refuted by Pearce and Eisenbrey,<sup>10</sup> and by Edmunds.<sup>6</sup>

While the best evidence indicates that the fall in blood pressure in anaphylactic shock in the dog is due to injury of the nerve endings in the vessel walls, it seemed desirable to determine also the condition of the sympathetic nervous system, a possible factor which has hitherto been more or less completely neglected. In the course of experiments conducted for this purpose results were obtained which were thought to be of sufficient interest to warrant their report.

A healthy dog was given a subcutaneous injection of 2 c.c. of normal horse serum. Three weeks later the animal was anesthetized with ether, and the carotid or femoral artery connected with a mercury manometer. The normal reactions of the animal to intravenous injections of standard doses of epinephrin (0.5 c.c. of a 1:20,000, or 1 c.c. of a 1:50,000 solution) and of nicotin (0.5 c.c. of a 1:2000, or 1 c.c. of a 1:4000 solution), were first obtained. These reactions were shown by Hoskins and Wheelon<sup>11</sup> to be "sufficiently constant for a given animal to permit their use as criteria of activity and irritability of the sympathetic nervous system."

After the normal reactions had been recorded, the animal was given from 4 to 6 c.c. of normal horse serum intravenously, the serum being washed into the vein with from 6 to 8 c.c. of normal salt solution. There was an immediate slight rise in pressure, due to the volume of fluid injected, with a prompt return to normal. Within from 10 to 30 seconds in nearly all the animals the blood pressure began to fall and, in about the same period of time, it had reached its lowest level. At intervals of from 1 to 3 minutes thereafter intravenous injections of standard doses of epinephrin and nicotin were given. In a few animals barium chlorid and pituitrin were also used, the former producing in none of my animals the prompt recovery mentioned by Biedl and Kraus.

The amount of fall in pressure varied, but usually amounted to from 50 to 80 mm. of mercury. In fatal cases pressures as low as 20 to 30 mm. of mercury were obtained. In such animals respiration stopped while the heart was still active. None of these fatal cases is included in the series here reported. The duration of the period of low blood pressure varied from a few minutes to more than 3 hours in one instance (Dog A 10. Chart 2).

Altogether 11 animals were thus observed. The results in 2 animals are shown in Charts 1 and 2. From these and other tracings not presented the following facts are evident:

<sup>8</sup> Jour. Pharm. and Exper. Therap., 1912, 3, p. 167.

<sup>9</sup> Skandin. Arch. f. Physiol., 1914, 31, p. 388. Abstracted in Zentralbl. f. Physiol., 1914, 29, p. 15.

<sup>10</sup> Jour. Pharm. and Exper. Therap., 1912-13, 4, p. 21.

<sup>11</sup> Am. Jour. Physiol., 1914, 34, p. 81.

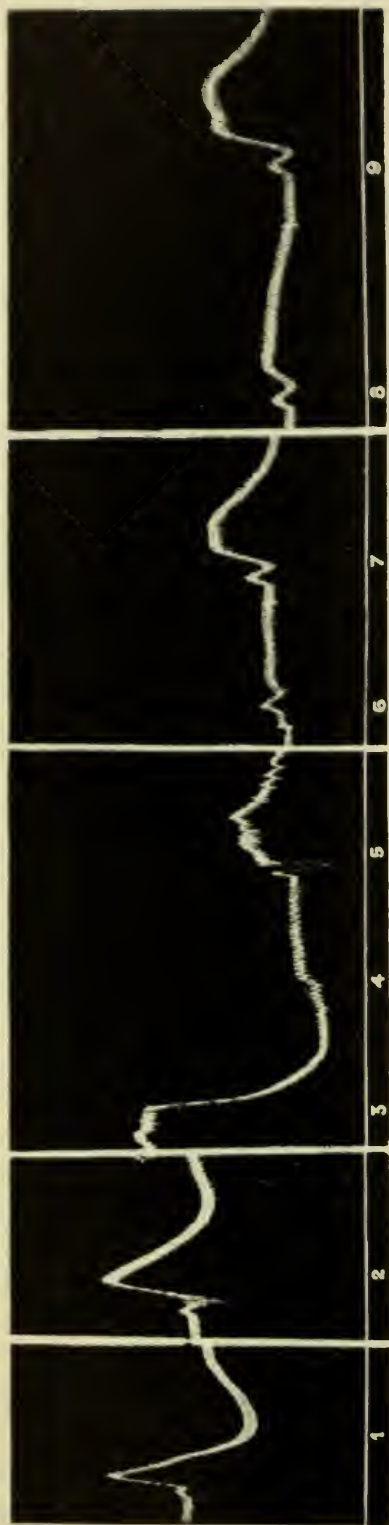


CHART 1

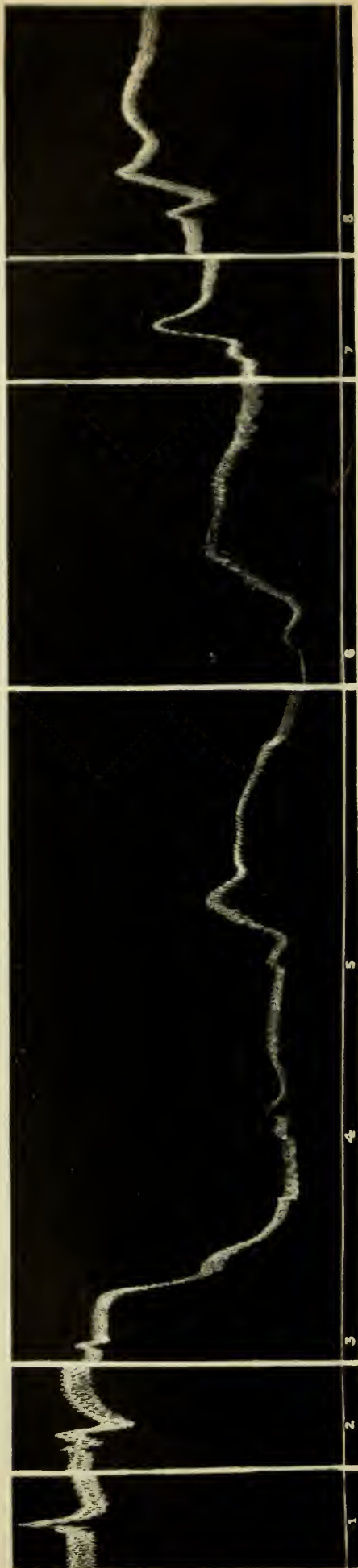


CHART 2

#### CHART 1

Dog A8. Given subcutaneous injection of 2 c.c. of normal horse serum, Nov. 25, 1914. Anaphylactic shock induced by intravenous injection of 6 c.c. of normal horse serum, Dec. 16, 1914.

1. Normal reaction to 0.5 c.c. of a 1:20,000 adrenalin solution.
2. Normal reaction to 0.5 c.c. of a 1:2000 nicotin solution.
3. Intravenous injection of 6 c.c. of normal horse serum at 3:05 p. m.
4. 0.5 c.c. of adrenalin solution at 3:07 p. m.
5. 0.5 c.c. of nicotin solution at 3:08 p. m. Marked dyspnea.
6. 0.5 c.c. of adrenalin solution at 3:45 p. m.
7. 0.5 c.c. of nicotin solution at 3:47 p. m. Moderate dyspnea.
8. 0.5 c.c. of adrenalin solution at 4:19 p. m.
9. 0.5 c.c. of nicotin solution at 4:24 p. m. Moderate dyspnea.

#### CHART 2

Dog A10. Given subcutaneous injection of 2 c.c. of normal horse serum, Dec. 19, 1914. Anaphylactic shock induced by intravenous injection of 7 c.c. of normal horse serum, Jan. 9, 1915.

1. Normal reaction to 0.5 c.c. of a 1:20,000 adrenalin solution.
2. Normal reaction to 0.8 c.c. of a 1:2000 nicotin solution.
3. Intravenous injection of 7 c.c. of normal horse serum at 11:16 a. m.
4. 0.5 c.c. of adrenalin solution at 11:19 a. m.
5. 0.8 c.c. of nicotin solution at 11:21. Dyspnea.
6. 0.8 c.c. of nicotin solution at 11:29 a. m. Animal was practically moribund when the injection was made. Marked dyspnea followed. Blood pressure rose from 26 to 88 mm. of mercury.
7. 0.5 c.c. of adrenalin solution at 11:36 a. m., 20 minutes after onset of shock.
8. 0.8 c.c. of nicotin solution at 11:41 a. m. Pressure reached normal level 36 minutes after onset of shock.

1. Immediately after the blood pressure has reached its lowest level, there is no response to injections of either epinephrin or nicotin

2. As time elapses, the reaction to nicotin reappears before the response to epinephrin. This may occur before the blood pressure begins to show any upward trend.

3. The ability to react to injections of nicotin returns more rapidly than the ability to react to epinephrin, and, in most instances, becomes markedly exaggerated. This usually takes place at a time when the response to epinephrin is still weak or even absent.

In producing vasoconstriction and consequent rise in blood pressure, pituitrin is believed to act directly on the muscular coat of the vessel walls while epinephrin stimulates the nerve endings.<sup>12</sup> The pressor effect of nicotin was shown by Hoskins and Ranson<sup>13</sup> to be "due about half to a stimulation of the vaso-constrictor (vaso-reflex?) center proper, and half to stimulation of the ganglion cells."

If the fall in blood pressure in anaphylactic shock in the dog is due to a paralysis of, or injury to, the nerve endings in the vessel walls, as suggested by Biedl and Kraus and by Pearce and Eisenbrey, it is difficult to account for the fact that there is an earlier return of reaction to nicotin than to epinephrin. The continued low pressure is due to the accumulation of blood in the large veins and vessels of the splanchnic area so that too little blood reaches the heart to enable it to keep up the arterial pressure. The action of nicotin must in some way reduce the amount of blood in these areas and increase the volume which reaches the heart. There are two possible ways in which this might be accomplished: (1) by causing a vasoconstriction of the vessels concerned; and (2) by action on respiration.

It has been shown that at a time when the reaction to epinephrin is very weak the response to nicotin may be greatly exaggerated. Hoskins, Rowley, and Rosser<sup>14</sup> found that in conditions of low pressure due to hemorrhage, the reaction to nicotin was augmented. In their animals the response to epinephrin remained normal. Since they observed the same phenomena in otherwise normal animals with both carotids ligated, they concluded that these results were due to increased irritability of the vasomotor center brought about by anemia of that center. In the condition of anaphylactic shock, however, the pressor effect of epinephrin is either absent or greatly diminished at a time

<sup>12</sup> Pearce and Eisenbrey: *Arch. Int. Med.*, 1910, 6, p. 218. Brodie and Dixon: *Jour. Physiol.*, 1904, 30, p. 476.

<sup>13</sup> *Jour. Pharm. and Exper. Therap.*, 1915, 7, p. 375.

<sup>14</sup> *Arch. Int. Med.*, 1915, 16, p. 456.

when nicotin may cause an exaggerated rise in pressure. In other words, a pressor drug which acts on the nerve endings in the vessel walls produces no effect or one greatly reduced, while the pressor effect of a drug which acts on the vasomotor center and on the ganglion cells is increased. This is especially well demonstrated in Chart 2. This animal (A10) was apparently moribund when the injection of a standard dose of nicotin caused a rise of 60 mm. of mercury, and the animal ultimately recovered from the shock.

In explanation of this anomalous reaction, it is possible that the vasomotor center (including the sympathetic ganglia) is in a state of increased irritability. This condition may be caused by anemia of the center due to the low blood pressure. It was not due to occlusion of one of the carotids by connection with the manometer, for similar results were obtained when the manometer was connected with the femoral artery and the carotids left untouched. Whether the toxin (anaphylatoxin) which causes the shock may also increase the irritability of the vasomotor center, from the facts at hand can neither be affirmed nor denied. It does not appear necessary to presuppose such an action in order to explain the facts.

In this connection it is interesting to note that Beifeld, Wheelon, and Lovelette<sup>15</sup> recently reported studies on the influence of hypotensive gland extracts on vasomotor irritability. After injections of extracts of pancreas and salivary glands they obtained a fall in blood pressure with the same characteristics as the low pressure in anaphylactic shock, namely, a decreased reaction to epinephrin and an augmented reaction to nicotin. They conclude that "such extracts cause therefore, an augmented irritability of the vasoconstrictor center."

In producing a rise in blood pressure, epinephrin, reaching the nerve endings in the vessel walls by way of the blood itself, is a stimulus of approximately the same force whether the drug be injected before or after shock has been induced: (It is possible that on account of the great slowing of the circulation the concentration of the epinephrin in the blood in a given organ is less than would normally occur if it were mixed quickly by rapidly flowing blood.) But if there is a state of increased vasomotor irritability in anaphylactic shock, the pressor effect of a standard dose of nicotin would be increased. Such a stimulus might be able, therefore, to overcome the torpor or partial paralysis of the nerve endings in the vessel walls and even to produce an augmented rise in pressure.

<sup>15</sup> Amer. Jour. Physiol., 1916, 40, p. 360.

There is the further possibility that of two stimuli which reach a vessel wall, that one which arrives by way of a nerve, as does the stimulus of nicotin, may be better able to produce its effect under adverse conditions, than a chemical stimulus which reaches the vessel wall through the blood stream.

If there is a state of increased vasomotor irritability in anaphylactic shock it does not develop immediately, but only after the lapse of a few minutes. Whether the failure to obtain any reaction to either epinephrin or nicotin immediately after the onset of shock is due to a sudden complete paralysis of the whole vasomotor apparatus with more rapid recovery of the central elements, or to so complete a paralysis of the nerve endings in the vessel walls that even the exalted stimulating power of nicotin is unable to overcome it, or, finally, to the necessity of a sort of incubation period for the full development of increased irritability of the vasomotor center, cannot at present be definitely determined.

There is a second possibility to account for this action of nicotin, namely, its effect on respiration. In the doses used in these experiments, the usual effect was to increase the rate and depth of respiration, sometimes almost to the point of dyspnea. This lasted on an average for about 30 seconds. The negative pressure in the thorax and therefore the suction on the blood in the great veins must have been much increased during this period. It is not difficult to conceive that the pressure of the diaphragm on the abdominal organs coupled with the increased suction on the blood in the vena cava would bring a greater volume to the right side of the heart. Blood in the liver and overfilled veins of the splanchnic area is in the most favorable location to be drawn into the heart under these conditions. Thus it is possible that nicotin does not, in this instance, raise the blood pressure by the usual mechanism at all.

There is no evidence in the experiments here reported of any constriction of the vessels of the lungs as claimed by Schultz.<sup>7</sup> For the blood that reaches the right side of the heart as a result of the injection of nicotin is evidently quickly and easily forced through the lungs and on into the aorta, raising the arterial pressure.

In further support of the view that the effect of nicotin on blood pressure in anaphylactic shock may be due to its action on respiration, it should be noted that Dog A 10 during the period of falling pressure became dyspneic, as shown in Chart 2. During this time the pressure either ceased to fall or the decline at least became much less rapid.

The cause of this dyspnea is not known. No other animal in this series showed it, altho it was observed once in an animal in peptone shock.

Furthermore, in most of the animals, the injections of nicotin given immediately after the onset of shock did not have any appreciable effect on the respiration. It was only when dyspnea followed the injection of a standard dose of nicotin that the rise in blood pressure occurred. Animal A 10 is unique in that the first injection of nicotin after the onset of shock caused violent respiratory efforts associated with a rise in blood pressure. This problem will be discussed more in detail in another connection.

The fact that when no dyspnea was produced by an injection of nicotin the effect on the blood pressure was less than the normal response for that animal is suggestive evidence that in anaphylactic shock in the dog there is a reduced irritability of the sympathetic nervous system and possibly of the vasomotor center.

#### CONCLUSIONS

Anaphylactic shock in the dog is associated with a fall in blood pressure. During the period of low pressure the reaction to injections of epinephrin is either absent or greatly diminished, while the response to nicotin may be augmented. It is believed that the mechanism of the latter is chiefly dependent on its effect on respiration, and only to a very limited extent or not at all on direct stimulation of the vasomotor center or the sympathetic ganglia. There is evidence for the belief that there is present a condition of decreased irritability of the sympathetic ganglia and possibly of the vasomotor center. The prompt rise in arterial pressure after injections of nicotin associated with dyspnea is evidence that in anaphylactic shock in the dog there is no constriction of the vessels of the lungs.

# THE NECESSITY OF A STANDARD BLOOD-AGAR PLATE FOR THE DETERMINATION OF HEMOLYSIS BY STREPTOCOCCI\*

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The manner of growth of streptococci on blood-agar plates, as first suggested by Schottmüller, has been found a valuable means of differentiation. Schottmüller<sup>1</sup> found that by growth on a mixture composed of 2 c.c. of defibrinated human blood and 5 c.c. of plain agar, streptococci can be differentiated into 3 groups, which he describes as follows: *Streptococcus longus seu erysipelatos*, which appears after from 18 to 24 hours' incubation as a grayish circular colony surrounded by a zone of hemolysis from 2 to 3 mm. in diameter; *Streptococcus mitior seu viridans*, which appears after 24 hours' incubation as a very small greenish colony, no hemolysis being present except where the medium is very thin; and *Streptococcus mucosus*, which appears after 24 hours' incubation as a mucoid greenish very moist growth. These results have been investigated by a great number of workers and Schumacher,<sup>2</sup> Silverstrom,<sup>3</sup> Mandelbaum<sup>4</sup> and many others have accepted the method in general as a valuable means of differentiation. On the other hand Bietske and Rosenthal<sup>5</sup> claimed that hemolytic strains could be changed into nonhemolytic and vice versa. Mandelbaum<sup>4</sup> ascribes the different results to the use of different media. He found that on blood-agar plates made according to the formula of Schottmüller a type of hemolysis is produced by *Streptococcus mitior* which he describes as follows: "hemolytic zone in *mitior* appears rarely in 24 hours, most often at 48 to 96 hours' incubation; microscopically one finds blood cells directly beneath the colony, while many discolored cells remain in the clear zone becoming more abundant further away from the colony." Smith and Brown<sup>6</sup> have called atten-

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<sup>1</sup> München. Med. Wchnschr., 1903, 50, p. 849.

<sup>2</sup> Centralbl. f. Bakteriöl., I, O., 1906, 41, pp. 628, 712.

<sup>3</sup> Ibid., p. 409.

<sup>4</sup> Ztschr. f. Hyg., u. Infektionskrankh., 1908, 58, p. 26.

<sup>5</sup> Arb. a. d. path. Inst., Berlin, 1906.

<sup>6</sup> Jour. Med. Research, 1914, 31, p. 455.

tion to a similar variation in hemolysis and have designated the distinct hemolysis as Type Beta and the indistinct as Type Alpha.

Little attention seems to have been paid to Mandelbaum's work in this country. Recorded results in the determination of hemolysis on blood agar are unsatisfactory and difficult of comparison. Defibrinated and citrated human, horse, rabbit, sheep, and goat blood in varying amounts are used in the preparation of blood-agar plates and the results are read after from 24 to 96 hours' incubation. Von Lingelsheim<sup>7</sup> has called attention to the necessity of a standard technic in the determination of hemolysis, and more recently Holman<sup>8</sup> has proposed a medium composed of 5 c.c. of defibrinated human blood to 100 c.c. of plain agar. Holman also directs attention to variations in hemolysis due to other factors, as period of incubation, salt concentration of the agar base, temperature of the agar to which the blood is added, and the age of the blood-agar plates. It is evident that in order to secure data of at all comparable significance a standard blood-agar plate must be used.

In the work here reported, 5 strains of streptococci were studied on blood-agar plates of varying composition.

Organism 1 was a strongly hemolytic long-chained streptococcus isolated from the skin of a case of erysipelas; Organism 2 was a hemolytic strain from a scarlatinal sore throat; Organism 3 was a long-chained hemolytic streptococcus obtained from the tonsils in a case of rheumatic fever; Organism 4 was a nonhemolytic short-chained streptococcus from the sputum in a case of pneumonia; Organism 5 was a green-producing streptococcus obtained from the same tonsils as 3. Organisms 1 and 5 fermented saccharose, lactose, and maltose; Organism 2 fermented lactose, mannite, raffinose, maltose, and dextrose; Organism 3 fermented saccharose, lactose, maltose, and dextrose; Organism 4 fermented maltose only.

Each of these organisms was repeatedly transferred from a single colony on human defibrinated blood agar plates to ascites dextrose broth and again plated out in order to insure a pure strain. The inoculation was made by streaking a drop of a 4- to 6-hour ascites-dextrose-broth culture on the freshly prepared plates. The plates were studied after 24 hours' incubation at 37 C., after 48 hours, and after 96 hours. The fundamental conditions were kept as nearly uniform as possible. Thus, all the agar used was of one lot; a portion was made 1% alkaline with sodium hydrate, another portion 1% acid with hydrochloric acid, and a third portion of standard agar was made up with 1% dextrose. Blood was used unless otherwise mentioned in amounts of 1 c.c. of defibrinated blood or 2 c.c. of citrated blood to 6 c.c. of standard agar. Citrated blood was blood collected by aspiration from a vein into an equal quantity of 2% sodium citrate in physiologic salt solution.

<sup>7</sup> Kolle and Wassermann, *Handb. d. pathogen. Mikroorganismen*, 1912, 4, p. 463.

<sup>8</sup> *Jour. Med. Research*, 1916, 34, p. 377.

The observations with respect to hemolysis may be grouped under the following heads: (1) kind of medium; (2) kind of blood; (3) method of collecting blood; (4) amount of blood per plate; (5) number of colonies per plate; (6) period of incubation.

*Kind of Medium.*—Marked variations both macroscopically and microscopically occurred in the hemolytic zone on plain agar, alkaline agar, acid agar, and dextrose agar, to which blood in the proportion before mentioned had been added. Thus, no hemolysis was produced by any of the organisms in alkaline blood agar. Organism 1 produced a distinct hemolytic zone on plain human-defibrinated-blood agar, but an indistinct zone on the acid medium. With Organism 3, the results were the reverse. Organism 2 produced a distinct zone of hemolysis on plain defibrinated-sheep-blood agar and plain citrated-sheep-blood agar and on acid defibrinated-sheep-blood agar, but an indistinct zone of hemolysis on acid citrated-sheep-blood agar. Similar variations occurred in the case of other organisms. Variations in the width of the hemolytic zone also occurred. These irregularities did not appear to be dependent on the luxuriousness of the growth. Hemolysis was distinctly inhibited on dextrose blood agar, as has been shown by Lyall<sup>10</sup> and others. Organism 5 (viridans) gave practically equal amounts of greenish coloration on plain, acid, and alkaline agar, and more pronounced coloration on dextrose blood agar. Organism 2 produced a greenish color on dextrose defibrinated-human-blood agar, while no change was produced by Organisms 1, 3, 4, and 5. This, which is somewhat contradictory to the observations of Ruediger,<sup>9</sup> emphasizes the wide variations in this group.

*Kind of Blood.*—Blood of various species has been used, tho only a few comparative studies have been made. Silverstrom used defibrinated rabbit, horse, and human blood, but he mentions no marked differences. Guinea-pig blood has been reported by a number of observers<sup>7</sup> as unsuitable because of the rapid distintegration of the corpuscles. Kerner<sup>11</sup> found that the corpuscles of the dog are most easily hemolyzed, while human and frog corpuscles are the most resistant. In this study sheep, goat, horse, rabbit, and human bloods were used. Variations are especially marked in the microscopic appearance of the hemolytic zone. Organism 1 in 24 hours' incubation totally dissolved on a plain-agar base defibrinated sheep and horse corpuscles,

<sup>9</sup> Jour. Infect. Dis., 1906, 3, p. 663.

<sup>10</sup> Jour. Med. Research, 1914, 30, p. 515.

<sup>11</sup> Centralbl. f. Bakteriologie, I. O., 1905, 38, p. 223.

but did not dissolve goat, rabbit, or human corpuscles. Organism 2 dissolved citrated sheep corpuscles in acid agar, but not defibrinated sheep corpuscles in either acid or plain agar. Human cells were dissolved in acid media, while rabbit corpuscles were dissolved in plain agar, and horse corpuscles were not dissolved in any media. Organism 3 dissolved rabbit corpuscles only. Organism 4 produced hemolysis on horse blood only. Green-production by Organism 5 was most pronounced on sheep blood and least on human blood. Methemoglobin formed most quickly on rabbit blood.

*Method of Collecting Blood.*—Defibrinated or citrated blood is used by the majority of workers. Schumaker used defibrinated goat and human blood, which had been prevented from clotting by means of hirubin. He found no differences. Bernstein and Epstein<sup>12</sup> used ox blood, secured as follows: 400 c.c. of ox blood were collected in a sterile flask containing 30 c.c. of 1% ammonium oxalate in distilled water and 0.5 c.c. of 40% formalin. It was allowed to stand one-half hour, after which it was diluted with equal parts of 0.9% salt solution. Schottelius advised the use of fresh human blood, but gave no reason for its use. Citrated and defibrinated blood from rabbit, sheep, and man were used in this study. No striking macroscopic differences were observed. Microscopically, the celis did not dissolve as readily in citrated-blood agar as in defibrinated-blood agar. No regularity in this regard can be determined, however.

*Amount of Blood per Plate.*—Perhaps the most striking variations were seen with the use of various amounts of blood. Organism 1 produced a wider zone on medium containing 3 and 6 drops of human defibrinated blood than on that containing 9 and 12 drops. In the plates made with 3 and 6 drops the hemolysis was distinct, while on those made with 9 and 12 drops the hemolysis was indistinct. Organism 2 produced a distinct zone of hemolysis from 10 to 14 mm. wide on medium containing 3 and 6 drops of human defibrinated blood and a zone from 4 to 8 mm. wide on that containing 9 drops, while on medium containing 12 drops of blood it produced in 24 hours an indistinct zone of hemolysis from 4 to 8 mm. wide. Organism 3 produced a distinct zone of hemolysis from 1 to 3 mm. wide on plates containing 3, 6, and 9 drops of blood, while on the plate containing 12 drops of human defibrinated blood it produced indistinct hemolysis in a zone from 3 to 6 mm. in extent.

<sup>12</sup> Jour. Infect. Dis., 1906, 3, p. 772.

*Number of Colonies per Plate.*—On various occasions it was noticed that hemolysis was clear and distinct in case of isolated colonies, while indistinct and microscopically very different when the colonies were closely grouped. The reverse condition was noticed with other strains. In the case of *S. viridans*, green-production occurred in the form of a distinct narrow greenish ring when the colonies were isolated, while a diffuse discoloration occurred when the colonies were close together.

*Period of Incubation.*—Plates were read after 24, 48, and 96 hours' incubation at 37.5 C. In some cases the cells were dissolved after 48 hours when they were present at 24 hours. In the case of Organism 2 the distinct hemolytic zone at 24 hours' incubation, after 48 hours' incubation was totally obscured on rabbit and horse blood, and was indistinct on sheep and human blood because of the production of methemoglobin. The greenish coloration of *S. viridans* was usually more distinct at 48 hours' incubation, tho on rabbit blood a brownish color, instead of green, was produced at 96 hours' incubation. Organism 4 produced on horse blood at 96 hours' incubation a greenish zone indistinguishable from the colonies and plates of Organism 5 (*viridans*).

#### DISCUSSION

In the determination of a standard medium certain points must be kept in mind: (1) availability, (2) constancy of material, and (3) the necessary elements for proper growth of the organism. While this paper was under process of completion, Holman recommended as a standard medium for the determination of hemolysis, 5 c.c. of defibrinated human blood to 100 c.c. of agar, made up according to certain directions which he gives. As a standard agar has been used in water analysis for several years and is used in most laboratories in this country, it is hardly necessary to have a specific plain agar base, tho the agar base suggested by Holman is much easier to make and would probably serve the purpose. Human blood is the most readily available and resists hemolysis by weakly hemolytic strains the best. I believe the Holman medium contains too small a quantity of blood, the hemolysis appearing indistinct in many cases. Many freshly isolated strains grow very poorly on medium containing such a small amount of hemoglobin. Schottmüller originally used 2 c.c. of defibrinated human blood to 5 c.c. of agar. This I believe to be more blood than

is necessary and the results are not sufficiently superior to warrant its use.

The following technic for the preparation of a standard blood agar is suggested: Standard agar used in water analysis is placed in quantities of from 40 to 100 c.c. in flasks. These are heated until the agar is melted, and then cooled to 45-50 C. One cubic centimeter of defibrinated human blood is added for each 6 c.c. of the agar base and the whole thoroughly mixed. Approximately 7 c.c. are used for each plate. Surface streaks are made and results noted after 24 hours' incubation at 37 C. Isolated colonies only should be described.

On such plates, strains of streptococci, pneumococci, and gonococci can usually be readily grown and distinguished.

#### CONCLUSIONS

Marked variations occur in both the macroscopic and microscopic appearance of the hemolytic zone of streptococci on blood-agar plates.

Differentiation and comparison of strains of streptococci on blood agar of indefinite or varying composition is wholly unreliable and valueless. For this purpose a standard blood agar, such as that recommended in this article, should be used.

# A STUDY OF THE AGGLUTINATION AND CULTURAL RELATIONSHIP OF MEMBERS OF THE SO-CALLED STREPTOCOCCUS-VIRIDANS GROUP\*

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The increasing importance of the nonhemolytic types of streptococci, particularly in focal infections with systemic disease, led us to undertake this study. Such study seemed especially desirable as little information is available as to the immunity relationships of members of this group, most of the investigations having been limited to the determination of biologic characteristics.

The cultures studied included a series known to be pathogenic for man, having been isolated from the blood stream in endocarditis and from other definitely pathologic conditions. For comparison, a series of strains was isolated from the tonsils of healthy individuals. The fundamental criteria used in selecting the cultures were typical streptococcus morphology, especially when they were grown in broth, insolubility in bile or bile salts, and absence of hemolysis as determined by the addition of broth cultures to washed blood cells and the development of greenish color on blood-agar plates. These tests were repeated to eliminate any false negative results. A few strains not in this category were included for comparative purposes in bringing out certain special points.

The strains were then studied to determine their fermentative characteristics, inulin, mannite, raffinose, and salicin being used, and to determine their action on washed blood cells—that is, whether they produce methemoglobin or not.<sup>1</sup> The results with respect to fermentation are given in the table.

The determination of the action on washed blood cells did not yield satisfactory results. When all the cultures were tested at one time, varying grades of methemoglobin-production were present, giving an unbroken series of gradations between the two large groups, of

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<sup>1</sup> Lyll: Jour. Med. Research, 1914, 30, p. 487.

sharply positive or definitely negative. The results on different tests were in general alike. Individual false negative results, however, were not infrequent. On the other hand, there was an indication in some instances that an ability to produce methemoglobin which had been dormant, had developed. In some cases a slight discoloration of the supernatant fluid suggested traces of hemolysis as well as methemoglobin-production. With 3 cultures this brownish hemolysis was very distinct, tho one of the strains had had no action on washed cells in a test several months previously, and the other two had been described as green-producing types without hemolysis at the time of isolation.

The production of methemoglobin therefore did not sharply divide the nonhemolytic streptococci into 2 groups. The quantitative character of the results may have been due to the fact that the conditions for the production of methemoglobin were not satisfactory, especially as false negative results were not uncommon. Unless the reaction can be made more uniform and sharper in results, much of its value is lost. At least it cannot be used as the basis for a primary grouping of streptococci.

The agglutination tests were made with broth cultures and immune rabbit sera. In most instances it was not necessary to shake the cultures mechanically if the sediment in the broth was carefully avoided. A varying degree of spontaneous agglutination was encountered. This varied from day to day and in most instances could be overcome by shaking. One culture, Endocarditis 14, which was persistently and strongly self-agglutinating, could not be used even when shaken. The normal content of agglutinins was determined in each rabbit serum used and this value deducted in tabulating the results.

It may be of value to give the method used in producing the agglutinating sera, as we have been able, by this method, to produce serum of high titer with great uniformity. The injections were given intravenously and daily, unless Sunday intervened, according to the following scheme, suspensions heated to 53 C. being employed at first, and then live cultures. The strains were grown on agar slants with a drop of citrated horse blood on the surface. The dosage averaged as follows in terms of total growth on the agar slant: heated,  $\frac{1}{4}$ ,  $\frac{1}{2}$ ,  $\frac{3}{4}$ ,  $\frac{3}{4}$ ; live,  $\frac{1}{2}$ ,  $\frac{1}{2}$ , 1. The last dose was continued to make 2 weeks of injections. The rabbits were bled from 4 to 5 days after the last injection.

The agglutination results have been combined in one table, the percentage values of the results being given. There seems to be no need

TABLE I

RESULTS OF THE STUDY OF THE AGGLUTINATION AND CULTURAL RELATIONSHIP OF MEMBERS  
OF THE STREPTOCOCCUS VIRIDANS GROUP

Strains and Origin		Inulin	Mannite	Raffinose	Salicin	Blood
Epidocarditis....	1.....	—	—	—	+	+
	2.....	—	—	—	+	+
	3.....	—	—	—	+	+
	4.....	—	—	—	+	±
	5.....	+	—	+	..	—
	6.....	—	+	+	+	—
	7.....	+	—	+	+	±
	8.....	+	+	+	+	—
	9.....	—	—	+	—	—
	10.....	+	—	—	+	±
	11.....	—	—	—	—	+
	12.....	+	—	+	—	+
	13.....	+	—	+	+	±
	14.....	+	—	+	+	±
	15.....	—	—	—	+	±
Meningitis.....	1.....	—	+	—	+	H <sup>1</sup>
	2.....	—	—	—	—	±
Apical Abscess..	1.....	—	—	+	+	+
	2.....	—	—	—	+	—
	3.....	—	—	—	+	—
	4.....	—	—	+	—	+
	5.....	+	—	+	—	±
M. rheumaticus (Beattie).....		—	+	+	+	+
Acute rheumatism.....		—	+	—	+	H <sup>1</sup>
Rheumatism and pericarditis.....		+	—	—	+	±
Arthritis (foot).....		—	+	+	+	+
Prostate (arthritis).....		—	+	+	+	±
Furuncle (neck).....		—	—	+	+	—
Chronic bronchitis.....		—	—	+	+	H <sup>2</sup>
Tonsils, normal individuals (the initial indicates the individual; the number, the strain)	V3.....	—	—	+	—	—
	K1.....	+	—	+	+	+
	K2.....	—	—	+	+	—
	N1.....	—	—	+	+	—
	N2.....	—	—	+	—	—
	T1.....	—	—	—	—	+
	W1.....	+	—	—	+	+
	W2.....	+	—	+	+	—
	W3.....	+	—	+	+	—
	W4.....	+	—	+	+	—
	W5.....	+	—	+	+	—
	H1.....	+	—	—	—	+
	H4.....	+	—	—	—	+
	H5.....	—	—	+	+	—
	B2.....	+	—	+	+	+
	B3.....	—	—	—	—	+
	B1.....	—	—	+	+	+
	M2.....	—	—	+	—	+
	M5.....	+	—	+	+	—
	S3.....	+	—	+	+	—
	S4.....	—	—	+	+	—
	S5.....	—	—	—	—	+
	Ta2.....	—	—	+	+	+
	Ta3.....	+	—	—	—	+
	Ta4.....	+	—	—	—	+
Titer of sera.....		..	..	..	..	..

## EXPLANATION OF TABLE

*Blood*.—In this column + means definite methemoglobin-production; ±, moderate methemoglobin-production; ±, slight or doubtful methemoglobin-production; —, no change; H, hemolysis (1) reddish brown, (2) deep garnet.

*Agglutination*.—To economize space and to make the results directly comparable and easy of interpretation, percentage values are given. The actual results were first reduced to numeric values according to the dilution results. Thus a complete reaction at 1:200 was given a value of 200. A half complete reaction was given a numeric value of half the dilution, and so on. The latter procedure was followed only in low dilutions, exceptionally in higher dilutions where a partial reaction persisted. In these instances the numeric



of giving all the reactions at the different dilutions of each serum separately. In the case of one serum the titer could not be determined because of spontaneous agglutination, but its action on the other strains is given because the results offer further evidence for deductions made in discussing results.

The agglutination reactions offered at least no basis for grouping of the strains of pathologic origin, tho cross agglutinations were present. The only possible exceptions were the endocarditis strains, Nos. 1, 2, and 3. These, which came to us in this order from Mt. Sinai Hospital, unfortunately were accompanied by no data concerning their origin. However, we feel that they were probably 3 successive isolations from the same case, repeated blood cultures being a common practice at this hospital.

A study of the cross agglutinations yields some interesting data. Serum immunized to Endocarditis Strain 10, as well as sera immunized to other strains, agglutinated Endocarditis Strain 11 to a slight degree, yet serum immunized to Endocarditis Strain 11 did not agglutinate Endocarditis Strain 10, nor the other apparently related strains. The most striking point is that certain strains were agglutinated to a varying degree by many sera, and this was more noticeable among the normal throat cultures. Furthermore, sera immunized to these strains from normal tonsils agglutinated, as a rule, a higher proportion of the tonsillar strains and commonly at much higher dilutions. The sera immunized to the tonsillar strains which were agglutinated by sera immunized to the types of pathologic origin, had no action with few exceptions (Endocarditis Strains 7, 9, and 11, and rheumatism, pericarditis, and furuncle strains) on the latter types. Thus serum immunized to Strain K 2, which was acted upon by most sera, did not agglutinate any of the strains of pathologic origin used in the preparation of those sera. In contrast to this, the three tonsillar strains, K 2, W 2, and M 5, showed a sharp degree of interagglutination. This would suggest a tendency to grouping, if one could explain the fact that they were acted upon by other sera when a reverse action was absent.

On the whole, the data seem to us to justify the conclusion that the types pathogenic for man constitute a heterogeneous group. At least from the practical standpoint, this conclusion seems justified; that is, the possibility of a multiplicity of groups is not excluded by the amount of work we have done. A few of the types from patho-

logic conditions, but many of the tonsillar types, tended to possess a common agglutinin-binding content but did not necessarily show a common agglutinogenic content, as they did not cause the production of agglutinins active against other strains unless these strains had given evidence, at least to some extent, of possessing a common agglutinin-binding content for several sera. This deduction seems warranted, tho certain factors must be considered in relation to the cross agglutinations. The tonsillar strains showed a greater tendency, numerically, to spontaneous agglutination and a somewhat higher proportion were acted upon by the normal agglutinins present in the rabbit sera. Many strains showed a partial or slight agglutination in a dilution of 1:10. A few strains were agglutinated in dilutions over 1:10, but only partially. These reactions are noted in the table. The majority of these high reactions occurred with the three sera from rabbits injected with the tonsillar strains. In other words the sera of these rabbits had a higher content of normal agglutinins.

The greater tendency, therefore, of the tonsillar strains to be agglutinated spontaneously, and the greater number of these strains agglutinated in the normal serum, probably contributed in producing the results. The instability of the suspension as evidenced by the tendency to spontaneous agglutination would reveal traces of agglutinins otherwise inactive. The fact that the three rabbits used for the tonsillar strains had a higher normal content of agglutinins is probably in part responsible for the great number of cross agglutinations; that is, having a high content of normal agglutinins, they may have responded quantitatively higher in producing group or common agglutinins. This would again be further intensified when they were acting on the unstable suspensions. Altho these factors probably explain the high figures, they do not invalidate the deduction already given.

The agglutination results are in accord with the enormous multiplicity of types which can be differentiated by the determination of fermentative characteristics. On the other hand, there is no correlation between interagglutinations and fermentative reactions, even where the former are very marked.

There is little in the voluminous literature on streptococcus differentiation that is available for comparison with our results. Floyd and Wolbach<sup>2</sup> determined the interagglutination only of cultures representative of cultural subgroups of the streptococcus group as a whole.

<sup>2</sup> Jour. Med. Research, 1914, 24, p. 493.

Kligler<sup>3</sup> also employed agglutination in the attempt to correlate the results of this reaction with fermentative characteristics, studying the streptococcus group as a whole. He used sera prepared against 4 representative strains. Serum immunized to one endocarditis strain agglutinated only its homologous strain. A serum immunized to a strain from a normal tonsil agglutinated to some extent, strains from endocarditis, 2 from pleurisy, 1 from a lymph node in scarlet fever, and 1 from an antrum slightly. The only striking cross agglutination was with a strain from acute tonsillitis. We feel that the lack of ability of this last strain to cause hemolysis places it in all probability among the nonpathogenic mouth types. If this is so, he has demonstrated a sharp cross reaction similar to our own. The degree of reverse agglutination was not determined in any case.

#### SUMMARY

Streptococci of the "viridans" type isolated from pathologic conditions apparently constitute a heterogeneous group. Saprophytic types may show some relationship one to the other, but practically none to the pathogenic type. Therapy, serum or vaccine, to elicit specific results is therefore possible only with autogenous strains.

<sup>3</sup> Jour. Infect. Dis., 1915, 16, p. 327.

# STREPTOTHRIX IN BRONCHOPNEUMONIA OF RATS SIMILAR TO THAT IN RAT-BITE FEVER \*

PLATES 18 TO 20

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Hektoen<sup>1</sup> has described the various lesions of bronchopneumonia in white rats, a disease which appears to be of frequent occurrence. The same lesions have also been observed in the gray rat. In the course of a study of this condition, especially as it occurs in the white rat, a streptothrix was found which may be of interest in connection with rat-bite fever in man.

Sixty white rats showing acute or chronic bronchopneumonia were examined. A long, fine, straight or wavy filamentous organism was observed in smear preparations or by dark-field illumination in 56. No branching was seen in these specimens. Three lesions in which the organism was not found, were chronic. It was most abundant in acute lesions. It is a gram-negative organism, nonacidfast, and it stains fairly distinctly with carbol-gentian-violet and Giemsa stain. It is distinguished from cilia by its greater length, more pointed ends, and its occasional wavy form. In the dark field I observed once or twice a slow worm-like motion of the filamentous organism, and once a cork-screw motion. The organism is barely visible in the tissues stained with methylene blue, but is distinct in tissues impregnated with silver nitrate according to Levaditi's method. Here, it appears more wavy and more fragmented than in any other preparations. It is seen inside the bronchi and sometimes in the surrounding tissue when the infection extends there. This organism was not observed in 24 normal rats. Short curved gram-negative organisms were seen in smears from the lesions of 3 rats; diplococci in 4 and diphtheroid bacilli in 1.

The rats after being killed with chloroform were generally washed with Harrington's solution and the lungs removed with sterile instruments. If the tissues were not taken out aseptically, the surfaces were seared, and the mucoid or purulent contents of the bronchi withdrawn by a sterile pipet. Pieces of the lung containing lesions were also used for inoculation. Anaerobic cultures (Wright's pyrogallic-acid and sodium-hydroxid method or liquid petrolatum) were made in ascitic agar containing tissue, ascitic fluid and tissue, and semicoagulated horse serum with and without tissue—the media commonly used

\* Received for publication August 16, 1916.

<sup>1</sup> Tr. Chicago Path. Soc., 1916, 10.

for growing spirochetes—but no spirochetes were cultivated. Aerobic and anaerobic cultures were also made on blood agar and the ordinary laboratory media.

A streptothrix resembling the organisms seen in the smear preparations from the lung was isolated from 20 rats, in pure growth 13 times. Cultures from 33 rats remained sterile. Why successful cultures were obtained from some rats and not from others is undetermined. The character of the lesion does not seem to make any difference. Successful cultures were more frequent in the case of sick rats and of lesions in which other bacteria were present.

The other organisms isolated from 22 rats were *Streptococcus viridans* (6), and *pyogenes* (1), *pneumococcus* (2), *staphylococcus* (1), diphtheroid bacilli (3), anaerobic spirilla (3), an unidentified profusely growing gram-negative bacillus (4), and the colon bacillus (2). The last two were probably contaminations, as they were isolated also from some of the normal rats. Seven of these were isolated from cultures in which the streptothrix did not grow. Successful cultures of the streptothrix were grown on human-blood agar or ascitic goat-blood agar.

#### CULTURAL CHARACTERISTICS

This streptothrix is a facultative anaerobe, growing best at 37 C. It seems to grow as well with air as without. Slight growth appears at room temperature after several days' incubation. Growth generally appears in from 48 to 72 hours, in subculture in 24, as discrete, colorless or grayish, pinpoint, circular, elevated colonies, with sharp margins. At first they appear dull, but later they become glistening. They may also increase slightly in size. The medium is not changed. The growth in the fluid of condensation and in ascitic broth is white and flocculent, the medium remaining clear. The growth on Loeffler's blood serum is similar to the growth on blood agar, but does not always occur, probably because of difference between the lots of media. Three strains were grown on various litmus sugar media, to which ascitic fluid had been added (1:4). Growth occurs in inulin, lactose, maltose, mannite, raffinose, saccharose, and salicin. A slightly acid reaction is produced by all in maltose and by 1 in salicin. There is no growth on potato, plain and dextrose agar, ascitic dextrose agar, in plain and dextrose broth, or in milk.

Cultures are viable at 37 C. for about 14 days after growth is established. When the organisms are first isolated they often die after a few days' incubation.

No streptothrix has been grown from the lungs of the normal rats examined.

#### MORPHOLOGY AND\* STAINING PROPERTIES

In cultures the organism is slender, straight or wavy, filamentous, varying greatly in length, and showing fragmentation and bacillus- and coccus-like forms. Some branching is seen in young cultures, but not with especial frequency. Spindle- or irregularly shaped swellings and involution forms are often observed. Twisted and wavy forms are more frequent on solid media than in ascitic broth, where the organisms appear generally as long threads or chains or as adherent masses of bacilli (Fig. 6). They do not show a radial

arrangement at the periphery of the colony. They stain with ordinary stains. They are gram-negative, except the swellings, and are not acidfast. The organism here is nonmotile.

#### ANTIBODY-PRODUCTION

An increase in opsonins and agglutinins for this organism has been observed in infected rats. For example, in one experiment the normal rat had a phagocytic index of 4, the two infected rats indexes of 12 and 20 respectively. The same control showed macroscopic agglutinations at 1:20, while the sera of the infected rats agglutinated at 1:60. The agglutination mixtures were incubated 1 hour and placed in the ice box over night.

#### PATHOGENICITY

Five young rats were each inoculated intraperitoneally with 2 c.c. of a mixture of a 24-hour growth in ascitic broth and that on 2 blood-agar slants. The streptothrix had been isolated from the lung of a rat dying spontaneously. On the following day 1 rat died, showing acute lesions in the lungs. The streptothrix was observed in smears from the heart blood and the lung, and was isolated from the blood and the lung. On the 2nd day after inoculation, another rat, which was sick, was killed. It showed pneumonia on both sides, the lesions being more advanced than those in the first rat. Smears from the lungs showed a few filamentous organisms. Cultures from the lung and the blood were negative. A third rat, killed on the 6th day, showed small acute lesions. The streptothrix was observed in smears from the lung. It grew slightly anaerobically on goat-blood agar. A fourth rat was killed on the 8th day. Very small lesions were present in the lungs and a few organisms were observed in smears. They grew but slightly. The fifth rat, killed on the 9th day after inoculation, showed no lesions.

Twelve young rats not inoculated were all normal.

Later, 2 rats were each inoculated intraperitoneally with the growth from 20 c.c. of an ascitic-broth culture of the same strain. One killed the following day showed acute congestion and hemorrhages of the lung and pleura. Smears from the lung showed rather short streptothrices. Smears from the blood were negative. The cultures from the lung showed *Streptococcus viridans*. The second rat was inoculated again 7 days later with the growth from 40 c.c. of an ascitic-broth culture. It was sick the following day and was killed, but no lesions were found.

Two guinea-pigs were each inoculated intraperitoneally with 5 c.c. of a 24-hour ascitic-broth culture of the streptothrix isolated from

the lung of inoculated Rat 1. Both guinea-pigs were ill the following day. One was killed, but no lesions in the lungs were found. There were small hemorrhages in the adrenals, which appeared swollen. The blood cultures were negative. The other guinea-pig was killed the next day. There was a collapsed spot in one lobe of the lung. No organisms were found. The cultures from the lung and blood were negative. Another guinea-pig inoculated intraperitoneally with the growth from 15 c.c. of an ascitic-broth culture remained well.

Two rabbits inoculated intravenously with the growth of the same organism on 2 blood-agar slants, died at the end of 3 and 4 weeks respectively. The lungs were normal.

A monkey (*Macacus rhesus*) was inoculated intravenously with the 24-hour growth from 200 c.c. of an ascitic-broth culture of another streptothrix, isolated from the lung of a rat dying spontaneously. The monkey's temperature rose the following day from 102.6 to 104.6+. The number of leukocytes rose from 8600 to 18,000. The monkey had a chill and diarrhea, and was very ill for 2 days. No eruption was observed. There was another rise in temperature with a slight increase in the number of leukocytes, affecting principally the polymorphonuclears, at the end of 4 weeks, and a similar rise in temperature and number of leukocytes at the end of 8 weeks. No eruption was seen and the monkey appeared perfectly well.

The anaerobic spirilla isolated from 3 rats were not pathogenic for white rats.

From these experiments the conclusion may be drawn that this streptothrix is slightly pathogenic for white rats, often producing acute lesions in the lungs, but is not pathogenic for rabbits or guinea-pigs. The organism has some pathogenicity for the monkey.

#### DISCUSSION

This streptothrix corresponds in general both morphologically and culturally with *Streptothrix muris-ratti*, isolated in pure culture from the blood of patients with rat-bite fever by Schottmüller<sup>2</sup> and Blake,<sup>3</sup> and seen in the blood during paroxysms by Tileston.<sup>4</sup> The descriptions of the organisms by the first two observers differ slightly. Schottmüller describes the color of the original colonies as grayish-black, without sharp edges, those in subculture on blood agar as grayish-white, and those on Loeffler's serum plates as colorless. Blake

<sup>2</sup> Dermat. Wehnschr., 1914, 58, Suppl., p. 77.

<sup>3</sup> Jour. Exper. Med., 1916, 23, p. 39.

<sup>4</sup> Jour. Am. Med. Assn., 1916, 66, p. 995.

describes the color of the colonies on Loeffler's blood serum as whitish, those on ascitic agar as grayish-white. His colonies are sharply margined. Schottmüller's organisms are "tinged bluish" with the Gram stain; Blake's are gram-negative. Blake gives a much fuller description of his organisms than Schottmüller. However, their organisms correspond so closely that it seems probable that they are identical.

Blake, who kindly examined 3 strains of the rat streptothrix, found that it differs from his organism in growing a little less abundantly on Loeffler's blood serum and in showing more beaded and coccus-like forms. He found the appearance of the colonies on solid media, the growth in ascitic broth, and the other characteristics identical. He was unable to demonstrate any agglutination of the strains by the serum of his rat-bite-fever patient. One strain of the rat streptothrix differs from his in producing slight acidity in salicin. Blake did not use maltose ascitic-fluid broth, which was slightly acidified by the three strains of the rat streptothrix tested.

Futaki<sup>5</sup> and his associates describe a motile spirochete in the skin and lymph node of 2 cases of rat-bite fever, but details of their experiments and cultures are not available. Some of the spiral forms in my Levaditi specimens closely resemble the spirochetes shown in their drawing.

According to Crohn<sup>6</sup> there is no record at hand of examination of the rat inflicting the injury. He states that white rats and mice are not known to cause rat-bite fever. I therefore examined 28 wild rats and found 1 infected. The lesions in the lung were the same as those observed in the white rats, and showed the same organism. As the trachea of infected rats is full of mucus containing large numbers of the organisms, it is readily seen that a bite from such a rat might be infectious.

#### CONCLUSIONS

A streptothrix similar to *Streptothrix muris-ratti*, isolated from the blood of patients with rat-bite fever, has been observed in smears and isolated in pure culture from the lungs of rats with bronchopneumonia.

An increase in opsonins and agglutinins for this organism has been found in infected rats.

Acute lesions have been produced in the lungs of rats inoculated intraperitoneally with cultures of this organism.

<sup>5</sup> Jour. Exper. Med., 1916, 23, p. 249.

<sup>6</sup> Arch. Int. Med., 1915, 15, p. 1014.

## EXPLANATION OF PLATES

### PLATE 18

FIG. 1.—Smear from lung. Carbol-gentian-violet.  $\times 1200$ .

FIG. 2.—Section through lung of rat with bronchopneumonia, showing the streptothrix. Levaditi preparation.  $\times 1200$ .

FIG. 3.—A 24-hour growth of the streptothrix on ascitic-fluid goat-blood agar. Carbol-gentian-violet.  $\times 1200$ .

### PLATE 19

FIG. 4.—Section through bronchus showing mucus filled with streptothrices. Levaditi preparation.  $\times 350$ .

FIG. 5.—Same as Fig. 4.  $\times 1200$

### PLATE 20

FIG. 6.—A 24-hour growth in ascitic broth. Carbol-gentian-violet.  $\times 1200$ .

FIG. 7.—A 24-hour growth on human-blood agar. Carbol-gentian-violet.  $\times 1200$ .

Figure 1

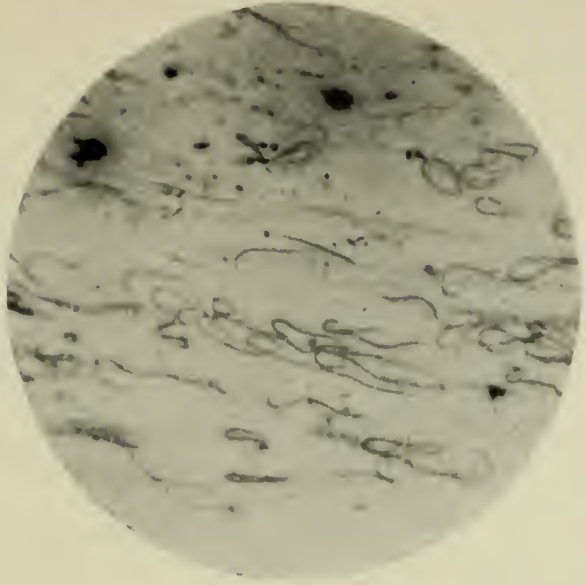


Figure 2

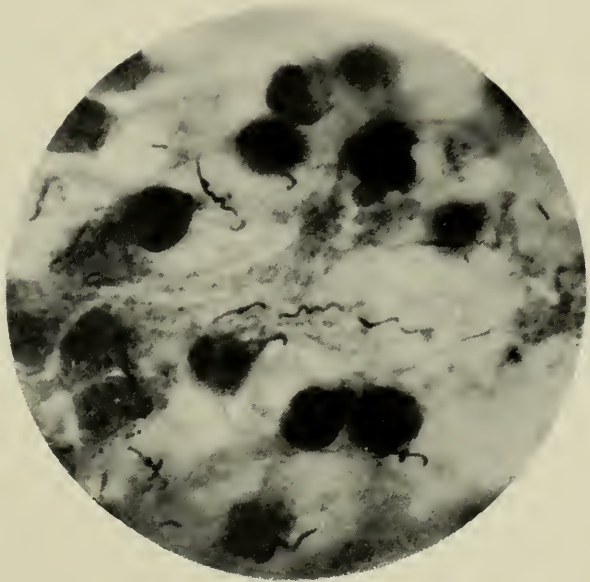


Figure 3





Figure 4

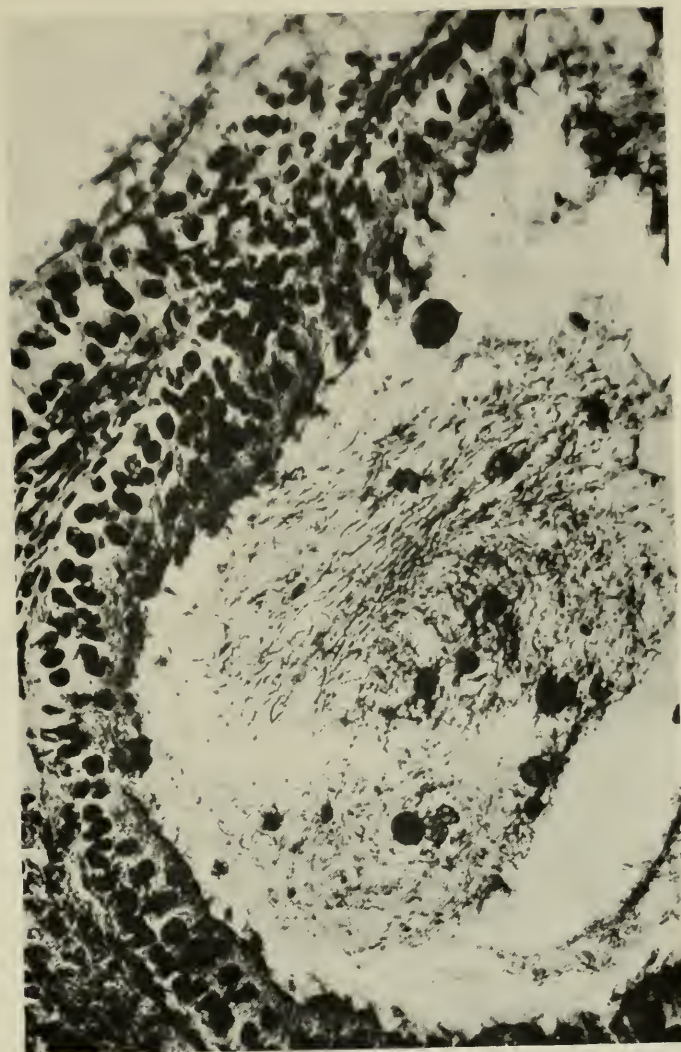


Figure 5

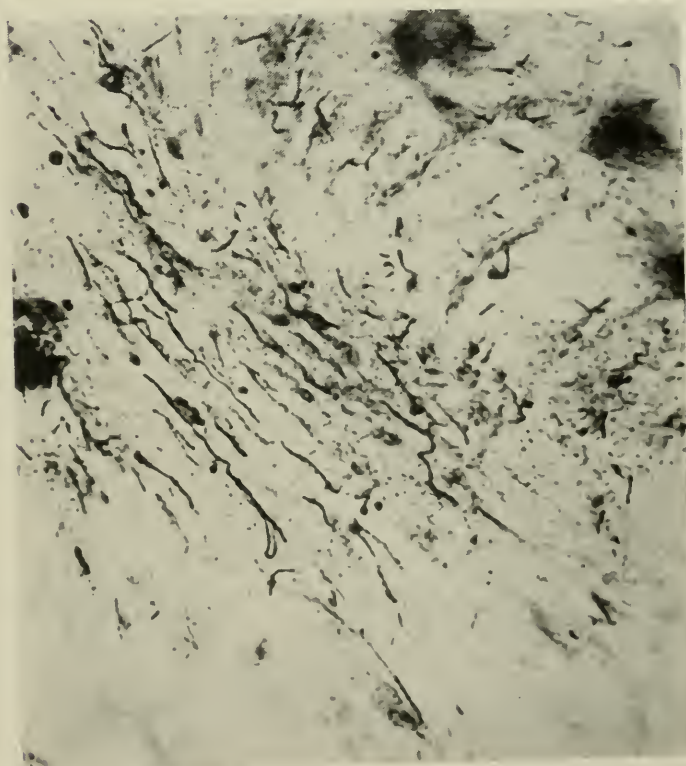




Figure 6

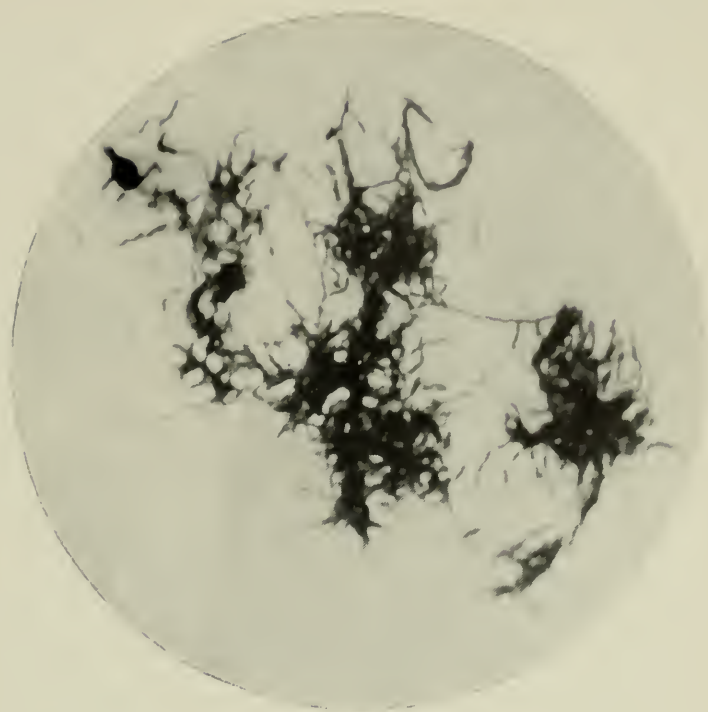


Figure 7





# ACID-PRODUCTION AND OTHER CHARACTERS OF BACILLUS-COLI-LIKE BACTERIA FROM FECES AND SEWAGE \*

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The ability to decompose carbohydrates with the formation of acid has long been recognized as one of the characteristics of *Bacillus coli*-like bacteria. This property of acid-production is the basis for the isolation of *B. coli* on the Wurtz litmus-lactose-agar plate, and also for the separation of *B. coli* from its relatives, *B. typhosus* and *B. paratyphosus*, on the Conradi-Drigalski agar medium. The ability to ferment various substances has been further utilized as a basis for the subdivision of the colon-aerogenes group. In these studies on classification, however, attention has been focused upon gas-formation rather than upon acid-production.

Browne,<sup>1</sup> in an extensive study of certain factors influencing acid-production, points out that *Bacillus coli*-like bacteria isolated from oysters formed less acid from carbohydrates than those isolated from human stools, and he attributed the difference to a loss of fermenting power by the organisms in their passage through sewage from the intestines to the oysters. Unfortunately, this investigator did not differentiate the different types of organisms with which he was working. It is entirely probable that the smaller amount of acid observed among the oyster strains was due to a greater incidence of some particular type or species of *Bacillus coli*-like microorganism rather than to a loss of fermenting power on the part of the intestinal forms. [After the completion of the experimental work for this paper, an article appeared by Clark and Lubs,<sup>2</sup> who pointed out that bovine fecal strains of *B. coli* give rise to a higher H<sup>+</sup>-ion concentration in glucose than do nonfecal (grain) strains.]

The present investigation was undertaken to determine the following:

1. Do *Bacillus coli*-like organisms from different sources (particularly animal sources) give rise to different amounts of acid in the

\* Received for publication August 30, 1916.

<sup>1</sup> Jour. Infect. Dis., 1914, 15, p. 580.

<sup>2</sup> Ibid., 1913, 17, p. 797.

decomposition of fermentable substances, and if they do, are the differences in acid-formation sufficiently great to warrant quantitative acid-production as a reliable differential index?

2. Is quantitative acid-production correlated with (a) the Mac-Conkey types,<sup>3</sup> (b) the Voges-Proskauer reaction, or (c) gas-formation?

3. Are the morphologic and physiologic characteristics correlated with the source?

#### CULTURES STUDIED

Altogether 167 organisms were studied; 156 were obtained from sewage and from the feces of horse, cow, sheep, pig, and man, and 11 were from the collection of the American Museum of Natural History.

The method of isolation has been described in a previous paper.<sup>4</sup> They were all of the colon-bacillus group; that is, gram-negative, usually short rods, which formed gas from glucose and lactose, coagulated milk, and did not liquefy gelatin in 20 days.

#### PREPARATION OF MEDIA

The medium employed for tests of acid-production consisted of 1% peptone water to which was added 1% of the test substance. Peptone water, rather than nutrient broth, was used, to eliminate the formation of acid from traces of any other fermentable substance which might be present in beef extract or meat infusion. The reaction of the medium was neutral to phenolphthalein.

*Sterilization.*—The medium was tubed (10 c.c. in Durham fermentation tubes) and sterilized in the autoclave for 10 minutes at 10 pounds pressure, which is a shorter period than is recommended in the Standard Methods for Water Analysis (1912). Immediately on removal from the autoclave the medium was rapidly cooled by immersion in cold water, then incubated for 2 or 3 days at 37 C. in order to eliminate tubes which had escaped proper sterilization. Nonsterile tubes were rarely found. Sufficient medium was prepared at one time to permit a test of all the cultures on one substance. Variations in the composition of the medium were reduced to a minimum by using distilled water and the same bottle of Witte's peptone throughout the work.

#### DETERMINATION OF ACID-PRODUCTION

Acid-production was determined in the following manner. A tube of peptone water was inoculated from an agar-slant stock culture and incubated at the body temperature (37 C.) for 24 hours. Two standard 4-mm. loops of this 24-hour peptone culture were then inoculated into each of 2 tubes of peptone medium containing the test substance and incubated for 36 hours at 37 C. Acid-production in duplicate tubes varied so little that duplicates were not employed with dulcitol, galactose, maltose, glycerol, and salicin.

<sup>3</sup> Jour. Hyg., 1905, 5, p. 333; 1909, 9, p. 86.

<sup>4</sup> Levine: Jour. Infect. Dis., 1916, 18, p. 358.

The body temperature was selected for incubation, because, as was shown by Browne,<sup>1</sup> acid-production by *B. coli* is most rapid at this temperature. He also showed that with certain carbohydrates and alcohols the maximal amount of acid is formed in less than 24 hours. Thirty-six hours' incubation was employed for convenience in this study. With the alcohol, glycerol, and the glucosid, salicin, the 36-hour incubation period was not sufficient. Acid- and gas-formation from these substances were therefore determined after 72 hours' growth.

*Titration.*—As the acidity of distilled water varied on different days, the following technic was adopted in order to obviate tedious subtractions of checks. To a pail of distilled water (6 to 8 liters) was added 1% phenolphthalein solution (5 gm. phenolphthalein in 1 liter of 50% alcohol). The water was boiled vigorously for 15 minutes and then neutralized with sodium hydroxid. Of this neutral distilled water, containing the indicator, 45 c.c. were dipped out into an evaporating dish or casserole, 5 c.c. of the test culture were added, and the amount of acid determined by titration with N/20 NaOH without boiling.

#### TREATMENT OF RESULTS

A few extremely high or low results will influence considerably the average acid-production of a collection of organisms. The use of unqualified averages may therefore lead to a misconception of the acid-producing properties of a group. To supplement the arithmetic mean, or numeric average, some statement should be made as to the distribution of the variates about the average. This may be indicated by the probable error or by the standard deviation. The coefficient of variability (the ratio of the standard deviation to the mean) is an excellent abstract measure of variability. The modal acid-production (the amount of acid most frequently formed) may, under certain conditions, be of greater significance than the average amount of acid formed.

In this study the mean, the probable error of a single variate, the standard deviation, the coefficient of variability, and the empirical mode are employed.

The standard deviation is the measure of variability most commonly employed, particularly by mathematicians. It may be expressed mathematically as

$$\sigma = \sqrt{\frac{\sum d^2 f}{n}}$$

where "n" is the number of variates, or observations, "d" the deviation of the individual variates from the mean, and "f" the frequency of a deviation "d". The standard deviation serves to indicate whether the departures from the mean are

small or great. The closer the individual organisms group themselves about the mean, or average, the smaller the standard deviation.

An example may make clear the meaning and significance of the standard deviation. Suppose that the amounts of acid formed by a group (A) of 4 organisms in glucose broth are 2.1, 2.2, 2.2, and 2.3% normal acid, and that those formed by another group (B) of 4 organisms are 1.9, 2, 2.4, and 2.5% normal acid. The average for each group is 2.2, but mere inspection shows that the organisms in Group A and those in Group B are quite differently distributed with respect to this average. In large collections of data inspection is impracticable, but the standard deviation serves well in its place. The standard deviation in Group A is  $\pm 0.07$  while for Group B it is  $\pm 0.25$ . The larger deviation in B denotes that the individuals in the group wander farther away from the average than do those in Group A.

The probable error is employed to indicate what confidence is to be placed in statistical results. The reliability of the mean and standard deviation may be determined by calculating their probable errors, but in this paper only the probable error of a single variate is considered. In a normal distribution the probable error of a single variate of a series of observations is defined as that departure from the mean, on either side, within which exactly one-half of the variates are found; that is, if in the study of acid-production by a large number of organisms, it is found that the mean (average) amount of acid formed is 2.25% normal, and that the probable error of a single observation is  $\pm 0.15$ , then half of the organisms have formed between 2.1% and 2.4% normal acid.

The coefficient of variability is the ratio of the standard deviation to the mean ( $\frac{\sigma}{M}$ ). It is an abstract measure of variability and may therefore be employed to advantage for comparing variability among different characters, or in the same character among different groups of organisms, particularly if their means differ widely.<sup>5</sup>

#### ACID-PRODUCTION IN SUBSTANCES FERMENTED BY ALL OF THE TEST ORGANISMS

Glucose, galactose, mannitol, maltose, and lactose were decomposed with gas-production by all strains.

##### A. GLUCOSE

The frequency distributions of the organisms with respect to acid-formation from glucose are shown in Table 1, where the relation of

<sup>5</sup> For a detailed description of these constants the reader is referred to *Principles of Breeding* (1907), by E. Davenport; *Statistical Methods* (1904), by C. B. Davenport; *Precision of Measurements* (1909), by Goodwin, and to an *Introduction to the Theory of Statistics* (1916), by Yule.

TABLE 1

RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION IN GLUCOSE

Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges- Proskauer	
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Neg- ative	Posi- tive
0.00-0.19													
0.20-0.39													
0.40-0.59													
0.60-0.79				1		1					1	1	
0.80-0.99				3		3					3	3	
1.00-1.19				2		1				1	2	1	1
1.20-1.39	3	4	3	1			3	5		3	11	9	2
1.40-1.59	4	3	9	3			5	2	1	11	19	15	4
1.60-1.79	8	5	3	6	2	1	2	7	4	6	22	20	2
1.80-1.99	28	8	10	8	5	5	9	8	14	13	54	54	
2.00-2.19	10	6	17	8	10	11	1	9	5	5	41	41	
2.20-2.39	1			2	2				1		3	3	
Total acid- formers.....	54	26	42	34	19	22	20	31	25	39	156	147	9
Mode.....	1.90	1.90	2.10	2.00	2.10	2.10	1.90	2.10	1.90	1.90	1.90	1.90	1.50
Mean.....	1.85	1.77	1.84	1.71	2.03	1.76	1.70	1.79	1.91	1.71	1.80	1.82	1.46
Probable error	±.14	±.18	±.19	±.29	±.11	±.32	±.17	±.18	±.11	±.18	±.20	±.20	±.12
Standard devi- ation.....	±.21	±.27	±.28	±.43	±.16	±.47	±.25	±.27	±.17	±.26	±.30	±.30	±.18
Coefficient of variation....	11.3	15.2	15.2	25.1	7.9	26.7	14.7	15.1	8.9	15.2	16.6	16.5	12.3

acid-production to the source, to the MacConkey types, and to the Voges-Proskauer reaction is also indicated.

The mode for acid-production by all strains is at 1.9% normal, with the mean at 1.8% normal acid.

The means or average quantities of acid formed by the MacConkey types indicate that Types III (communior) and I (acidi-lactici) produce about equal quantities of acid (1.84 and 1.85% normal respectively), while Type II (communis) forms somewhat less (1.77%), and Type IV (aerogenes) the least amount (1.71%). A comparison of Type IV, which forms the smallest quantity of acid, with Type I, which gives the greatest amount, indicates that the means tend to exaggerate the difference between the two types in ability to form acid from glucose. Type I has a well-defined mode at 1.90% and Type IV has a very indistinct mode at about the same point. The standard deviation in Type IV is  $\pm 0.43$ , or three times as great as the difference between the means of the two MacConkey types. Similar observations

may be made on the other types. It is therefore apparent that quantitative acid-production in glucose is not a reliable criterion for differentiation of the MacConkey types.

There are many irregularities in the frequency distributions of organisms from different sources with respect to acid-production in glucose. The organisms from horse and man group themselves in a manner simulating a normal distribution, but the frequency curves of those from cow, sheep, and pig, contain 2 modes. These multiple modes are probably due to the choice of classes and to the small number of cultures from each source. In the other test substances multiple modes are very infrequent. In the column headed "Mode," in the frequency tables, the primary mode is recorded.

The distribution of organisms from the sheep is interesting. Two distinct groups are indicated, one of which generally produces more than 2% normal acid and the other usually less than 1%. Of the 5 low-acid-formers, 4 are from a single animal (all the cultures obtained from that animal), and they are distinguished morphologically from all the other sheep strains in that they are distinctly longer.

Among the sewage strains 2 well-defined modes are evident, at 1.9% and 1.5% normal acid, corresponding with the modes of the Voges-Proskauer-negative and the Voges-Proskauer-positive organisms respectively.

In a consideration of the different animal sources it appears that the average amount of acid formed from glucose by *Bacillus-coli*-like organisms from horse (2.03%) is greater than the amounts formed by strains from pig, sheep and cow (1.70, 1.76, and 1.79%), while the amount formed by human strains is intermediate (1.91% normal). This relationship does not hold for other test substances and there does not seem to be any marked relation between the quantities of acid produced from glucose, and those formed from other fermentable carbohydrates or alcohols by colon-bacillus-like organisms from the animals recorded here.

Quantitative acid-formation is better correlated with the Voges-Proskauer reaction than with the source or with MacConkey's groups. The Voges-Proskauer-negative organisms give an average of 1.82% normal acid, with a mode at 1.90%, while the Voges-Proskauer-positive strains 1.46%, with a mode at 1.50% normal, and, altho the difference, 0.36, is probably not sufficient for reliable differentiation, it is nevertheless significant, because rather striking differences in acid-formation between the Voges-Proskauer-positive and the Voges-Pro-

kauer-negative strains are observed with many other test substances, as maltose, sucrose, glycerol, and dulcitol.

#### B. GALACTOSE

The frequency distributions with respect to acid-production in galactose are shown in Table 2. Less acid is formed from galactose than from glucose, and the frequency distributions are very nearly normal. Multiple modes are not present. The average amount of

TABLE 2

RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION IN GALACTOSE

Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges- Proskauer	
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Neg- ative	Posi- tive
0.00-0.19													
0.20-0.39				1			1				1	1	
0.40-0.59													
0.60-0.79				1						1	1	1	
0.80-0.99				2		1				1	2	1	1
1.00-1.19	3		4	6		3	1	2	1	6	13	8	5
1.20-1.39	28	18	21	10	8	6	12	17	19	15	77	76	1
1.40-1.59	21	8	16	13	11	11	6	11	5	14	58	57	1
1.60-1.79	1		1							2	2	1	1
1.80-1.99													
2.00-2.19													
Total acid- formers.....	53	26	42	33	19	21	19	30	25	39	154	145	9
Mode.....	1.30	1.30	1.30	1.50	1.50	1.50	1.30	1.30	1.30	1.30	1.30	1.30	1.10
Mean.....	1.37	1.36	1.37	1.27	1.42	1.36	1.35	1.36	1.33	1.34	1.35	1.36	1.21
Probable error	±.08	±.06	±.09	±.18	±.07	±.12	±.07	±.08	±.06	±.14	±.12	±.11	±.16
Standard devi- ation.....	±.12	±.09	±.13	±.27	±.10	±.18	±.11	±.12	±.09	±.20	±.17	±.16	±.23
Coefficient of variation....	8.8	6.6	9.5	21.2	7.1	13.2	8.2	8.8	6.8	14.9	12.6	11.8	19.0

acid formed by all strains is 1.35% normal, with a distinct mode at 1.30%. (Acid was not determined from 2 cultures, 1 from pig and 1 from sheep, which broke just before titration.)

The MacConkey types, I, II, and III, each have a mode at 1.30% normal acid, and means at 1.37, 1.36, and 1.37% normal acid respectively. Altho the mode for Type IV is 1.50% normal acid—somewhat higher than for the other types—the mean, 1.27%, is lower, a circumstance indicating a greater variability in Type IV. This greater

variability is indicated by the much larger standard deviation and coefficient of variability. MacConkey types, therefore, cannot be differentiated on the basis of quantitative acid-production in galactose as indicated by titration with phenolphthalein.

There does not seem to be any correlation between the amount of acid formed from galactose and the source of the organisms. One organism from the cow formed less than 0.4% acid. It was omitted in calculating acid-production by the group. If included, the mean for the cow strains becomes 1.30%, with a coefficient of variability of 19.2%.

The Voges-Proskauer-positive strains form somewhat less acid (1.21%) than do the Voges-Proskauer-negative strains (1.36%). The difference (0.15% normal) is slight, but it is greater than the differences observed with the MacConkey types or with the strains from different sources. The difference is of some interest, moreover; for, as will appear later, whereas the Voges-Proskauer-positive strains form less acid from the monosaccharids than do the Voges-Proskauer-negative strains, the reverse is true when more complex substances (except lactose) are fermented.

#### C. MANNITOL

The hexite, mannitol, is attacked about as readily as galactose. The average amount of acid formed by all strains is 1.32%, with a sharp mode at 1.30% normal. (Acid-production was not determined in 2 cultures, 1 from horse and 1 from man.) The frequency distributions and the relation of the Voges-Proskauer reaction, the source, and the MacConkey types to the amount of acid formed from mannitol are shown in Table 3.

The mode for each of the MacConkey types is at 1.30%, and the means are 1.32, 1.30, 1.33, and 1.31% normal acid respectively. The MacConkey types are therefore indistinguishable on the basis of quantitative acid-production from mannitol.

A comparison of the amounts of acid formed by Voges-Proskauer-negative and Voges-Proskauer-positive strains indicates that the latter attack mannitol somewhat more readily, but the difference is not appreciable.

Except for the horse strains, which have a mode at 1.50%, the organisms from all the other sources group themselves around 1.30% normal acid as a mode. In general, the differences observed between the means are too slight to be of any significance. The average of the

sheep strains is the lowest, 1.21%, as compared with 1.34% for human strains, 1.38% for horse, 1.36% for sewage, 1.31% for cow, and 1.28% normal for pig strains. The lower average of the sheep strains is due to the presence among them of a few low-acid-producing organisms rather than to a lesser ability of the group as a whole to attack mannitol. That sheep strains form acid from mannitol as readily as do those from the cow, pig, and man is indicated by the coincidence of their

TABLE 3

RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION IN MANNITOL

Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges-Proskauer	
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Neg-ative	Posi-tive
0.00-0.19													
0.20-0.39													
0.40-0.59													
0.60-0.79				2		2					2	2	
0.80-0.99	1			4	1	3		1			5	5	
1.00-1.19	9	6	7		1	3	5	6	3	4	22	22	
1.20-1.39	29	14	20	15	6	9	9	19	13	22	78	75	3
1.40-1.59	13	6	14	12	10	5	6	5	8	11	45	40	5
1.60-1.79	1									1	1	1	
1.80-1.99				1						1	1		1
2.00-2.19													
Total acid-formers.....	53	26	41	34	18	22	20	31	24	39	154	145	9
Mode.....	1.30	1.30	1.30	1.30	1.50	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.50
Mean.....	1.32	1.30	1.33	1.31	1.38	1.21	1.31	1.28	1.34	1.36	1.32	1.31	1.43
Probable error	±.10	±.09	±.09	±.17	±.11	±.18	±.10	±.09	±.09	±.12	±.12	±.12	±.12
Standard deviation.....	±.15	±.14	±.14	±.26	±.17	±.26	±.15	±.14	±.13	±.18	±.18	±.18	±.18
Coefficient of variation....	11.3	10.8	10.5	19.8	12.3	21.2	11.4	10.9	9.7	13.2	13.6	13.7	12.1

modes. The strains from the horse tend to form somewhat more acid than do those from other animals, but the difference is too slight to be of any differential significance.

#### D. LACTOSE

The amount of acid formed from the disaccharid, lactose, in 1% lactose peptone solution is very nearly the same as that formed from the monosaccharid, galactose, and from the hexite, mannitol. The mode for all strains is at 1.30%, and the mean is also at 1.30% normal acid. The frequency distributions are shown in Table 4.

MacConkey Type II has an ill-defined mode at 1.50%, and the mean is 1.25% normal acid. The mode for the other types (I, III, IV) is at 1.30%, and the means are 1.31, 1.32, and 1.33% normal acid respectively. The MacConkey types are indistinguishable on the basis of quantitative acid-production in lactose.

There is no evident relation between the source and the amount of acid produced from lactose.

TABLE 4  
RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION IN LACTOSE

Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges- Proskauer	
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Neg- ative	Posi- tive
0.00-0.19													
0.20-0.39		1					1				1	1	
0.40-0.59													
0.60-0.79		3					3				3	3	
0.80-0.99	1	1		6		5	1			2	8	7	1
1.00-1.19	13	1	11		4	1	1	6	12	1	25	24	1
1.20-1.39	30	9	19	15	10	3	11	18	11	20	73	67	6
1.40-1.59	8	11	10	10	5	9	3	7	2	13	39	38	1
1.60-1.79			2	3		4				1	5	5	
1.80-1.99	2									2	2	2	
2.00-2.19													
Total acid- formers.....	54	26	42	34	19	22	20	31	25	39	156	147	9
Mode.....	1.30	1.50	1.30	1.30	1.30	1.50	1.30	1.30	1.30	1.30	1.30	1.30	1.30
Mean.....	1.30	1.25	1.31	1.32	1.31	1.25	1.16	1.31	1.22	1.38	1.30	1.31	1.26
Probable error	±.12	±.22	±.11	±.16	±.09	±.19	±.22	±.09	±.09	±.13	±.15	±.15	±.11
Standard devi- ation.....	±.17	±.32	±.17	±.23	±.14	±.28	±.33	±.13	±.13	±.20	±.22	±.22	±.16
Coefficient of variation....	13.1	25.6	13.0	17.4	10.7	22.4	28.4	10.0	10.6	14.5	16.9	16.8	12.7

There is no distinction between the amounts of acid produced from lactose by Voges-Proskauer-positive and Voges-Proskauer-negative strains. Both groups have their modes at 1.30%, and the means are but slightly removed from the modes, being 1.26 and 1.31% normal acid respectively.

E. MALTOSE

Decomposition of the disaccharid, maltose, yields considerably less acid than the decomposition of the monosaccharids, glucose and galactose, the hexite, mannitol, or the disaccharid, lactose, mentioned. All

strains considered, the average acid-production was 0.77%, with a very distinct mode at 0.7% normal acid. The frequency distributions of the organisms from different sources, of the MacConkey types, and of the Voges-Proskauer reaction with respect to acid-production in maltose are shown in Table 5. One organism apparently fails to show acid but forms gas. This neutral reaction is presumed to be due to reversion.

TABLE 5

RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION IN MALTOSE

Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges- Proskauer	
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Neg- ative	Posi- tive
0.00-0.19		1								1	1	1	
0.20-0.39													
0.40-0.59													
0.60-0.79	7	2	6	1		3		3	6	4	16	16	
0.80-0.99	31	17	24	18	9	19	13	15	18	16	90	90	
1.00-1.19	10	6	8	9	9		3	12	1	8	33	31	2
1.20-1.39	6		2	4	1		4	1		6	12	8	4
1.40-1.59			2	2						4	4	1	3
1.60-1.79													
1.80-1.99													
2.00-2.19													
Total acid- formers.....	54	25	42	34	19	22	20	31	25	38	155	146	9
Mode.....	0.70	0.70	0.70	0.70	0.80	0.70	0.70	0.70	0.70	0.70	0.70	0.70	1.10
Mean.....	0.76	0.73	0.76	0.83	0.81	0.67	0.81	0.77	0.67	0.85	0.77	0.75	1.12
Probable error	±.11	±.07	±.13	±.13	±.08	±.05	±.11	±.09	±.08	±.15	±.11	±.09	±.10
Standard devi- ation.....	±.16	±.11	±.19	±.19	±.11	±.07	±.16	±.14	±.12	±.23	±.17	±.14	±.15
Coefficient of variation....	21.0	15.1	25.0	22.9	13.6	10.4	19.8	18.2	17.9	27.0	22.1	18.7	13.4

Each of the MacConkey types has a sharply defined mode at 0.70% normal. The means for the types are 0.76, 0.73, 0.76, and 0.83% normal acid respectively. There is no correlation between quantitative acid-formation from maltose and the MacConkey types.

The relation of acid-formation from maltose to the source of *Bacillus-coli*-like organisms is not at all striking. The mode for each source is at 0.7% normal acid. The trend of the variation among the strains from horse, cow, pig, and sewage, is beyond the mode, so that the means are 0.81, 0.81, 0.77, and 0.83% normal acid respec-

tively, while the strains from man and sheep vary in the other direction, lowering the averages to 0.67% for man and 0.68% for sheep. It should be pointed out that the relatively high average for sewage, 0.83%, is due to the presence of Voges-Proskauer-positive organisms. The average for the sewage strains exclusive of the Voges-Proskauer-positive organisms, is 0.73% normal acid. Acid-production in maltose can not be considered a reliable index for differentiation of *Bacillus coli*-like organisms from the sources studied.

There is a rather marked and distinct relation between quantitative acid-production in maltose and the Voges-Proskauer reaction. It appears from Table 5 that the Voges-Proskauer-negative strains occasionally form more than 1% acid, but usually less than 0.8%, while the Voges-Proskauer-positive strains usually form more than 1% and never less than 0.8% normal acid. The mode for the Voges-Proskauer-negative strains is at 0.70% and the mean at 0.78% normal acid. The mode and mean for the Voges-Proskauer-positive strains are 1.10 and 1.12% respectively.

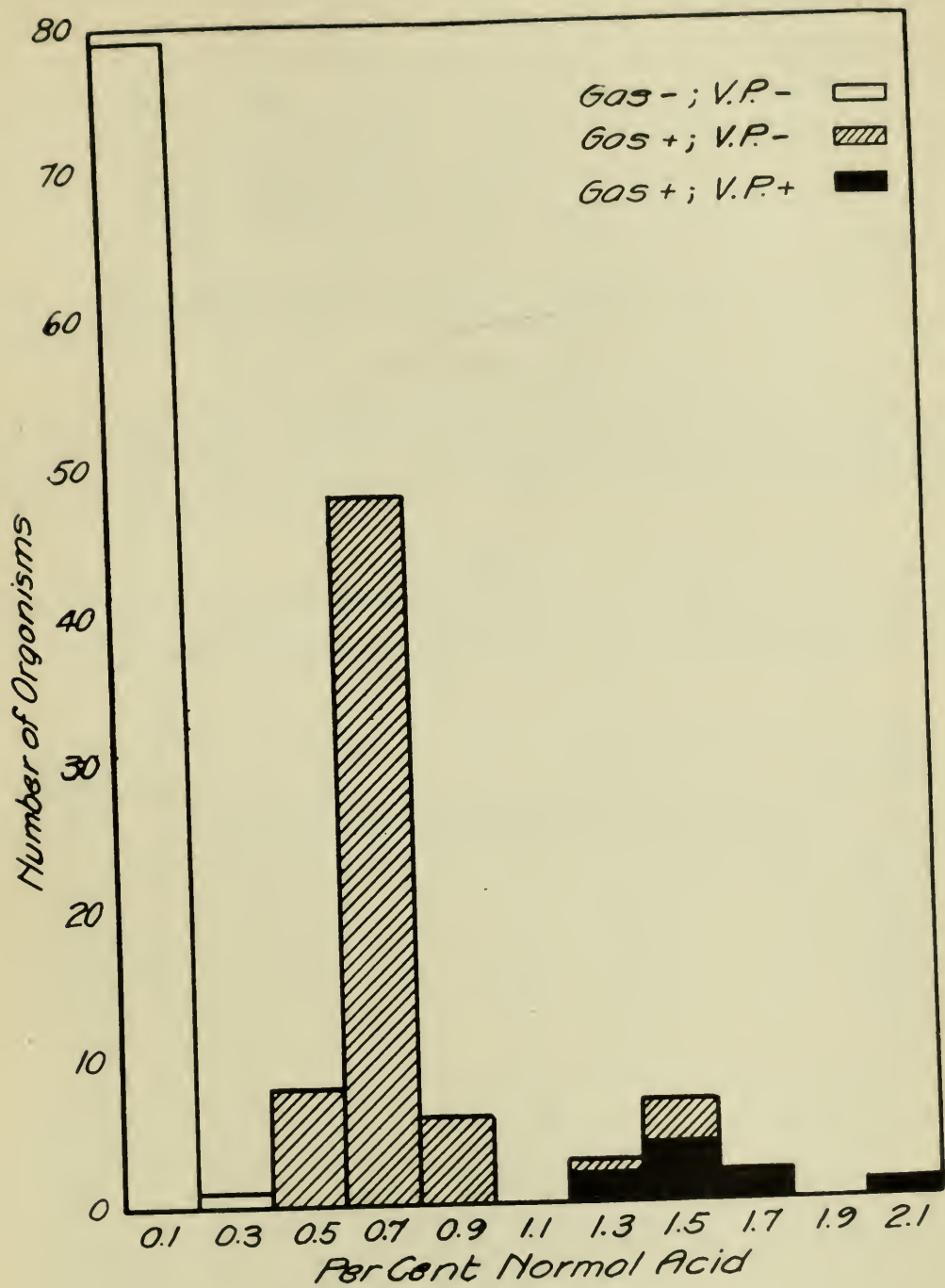
#### ACID-PRODUCTION IN SUBSTANCES NOT FERMENTED BY ALL THE TEST ORGANISMS

The disaccharid, sucrose, the trisaccharid, raffinose, the glucosid, salicin, and the alcohols, glycerol and dulcitol, were attacked by many, but not by all, of the organisms studied.

In calculating means and other constants for acid-formation, only those organisms which attacked the test substances were included. The line of demarcation for acid-production was selected at 0.4% normal acid, because in sucrose, raffinose, and dulcitol organisms which formed less than this amount in 36 hours at 37 C. rarely, if ever, formed gas, while those which produced more than 0.4% acid, practically always formed gas also.

#### A. SUCROSE

In Table 6 are shown the frequency distributions of acid-production in sucrose in relation to the MacConkey types, the source, and the Voges-Proskauer reaction. The relation of acid-production to gas-formation, and to the Voges-Proskauer reaction, is indicated, also, in Plot 1. (One organism, which was overrun in titration, is not included in the calculation.) Three modes are evident. One mode is at 0.1% normal acid and represents those organisms which do not form gas from sucrose. Acid-formation and gas-production in suc-



Plot I  
ACID-PRODUCTION FROM SUCROSE (All Strains)

rose are well correlated. Colon-bacilli-like organisms which form gas, also give rise to acid and vice versa. Among the gas-formers 2 groups are apparent; one forms acetylmethylcarbinol from glucose (V.P.+) and a relatively large amount of acid from sucrose (mode at 1.50% normal), while the other does not form acetylmethylcarbinol from glucose (V.P.—) and gives rise to a much smaller quantity of acid from sucrose (an extremely sharp and distinct mode at 0.70% normal).

MacConkey Types I and II do not form acid from sucrose. That Types III and IV are indistinguishable on the basis of quantitative acid-production in sucrose, is apparent from Table 6.

Ten of the organisms from cow, 15 from horse, 20 from sheep, 10 from pig, and only 3 from man, ferment sucrose. The amount of acid formed bears no definite relation to the animal source, but it should

TABLE 6  
RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION IN SUCROSE

Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges- Proskauer	
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Neg- ative	Posi- tive
0.00-0.19	53	26			4	1	10	21	21	22	79	79	
0.20-0.39	1								1		1	1	
0.40-0.59			3	5		3		2		3	8	8	
0.60-0.79			30	18	10	16	9	7	3	3	48	48	
0.80-0.99			4	2	2	1	1	1		1	6	6	
1.00-1.19													
1.20-1.39			1	2	1					2	3	1	2
1.40-1.59			3	4	2					5	7	3	4
1.60-1.79			1	1						2	2		2
1.80-1.99													
2.00-2.19				1						1	1		1
Total acid- formers.....	....	....	42	33	15	20	10	10	3	17	75	66	9
Mode.....	....	....	0.70	0.70	0.70	0.70	0.70	0.70	0.70	1.50	0.70	0.70	1.50
Mean.....	....	....	0.80	0.91	0.93	0.68	0.72	0.68	0.70	1.18	0.84	0.74	1.57
Probable error			±.18	±.27	±.20	±.06	±.04	±.07		±.33	±.23	±.14	±.16
Standard devi- ation.....			±.27	±.40	±.29	±.09	±.06	±.11		±.49	±.34	±.20	±.23
Coefficient of variation....	....	....	33.8	44.0	31.2	13.2	8.3	16.2		41.5	40.5	27.0	14.6

be noted that a few cultures among the horse strains form considerably more acid than any of the other animal strains. The high average for

the horse strains is due to the influence of these few cultures and is not a characteristic of horse strains in general.

The high average, 1.18% normal acid, of the 17 sewage strains which attacked sucrose, is due entirely to the presence among them of 9 Voges-Proskauer-positive organisms. The mean for the other 8 sewage strains is 0.75% normal acid.

Voges-Proskauer-negative strains attack sucrose less readily than the Voges-Proskauer-positive strains. The means for the two groups are 0.74 and 1.57%, and the empirical modes 0.7 and 1.5% normal acid respectively.

#### B. RAFFINOSE

The frequency distributions of the organisms with respect to acid-production in raffinose and the relation of acid-formation to the MacConkey types, to the source, and to the Voges-Proskauer reaction, are given in Table 7.

TABLE 7

RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION IN RAFFINOSE

Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges- Proskauer	
												Neg- ative	Posi- tive
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Neg- ative	Posi- tive
0.00-0.19	50	23			3		8	20	21	21	73	73	
0.20-0.39		1					1				1	1	
0.40-0.59	1		2	1	1	1		1		1	4	4	
0.60-0.79			13	5	5	4	2	5		2	18	18	
0.80-0.99	2	1	10	5	3	3	6	2	2	2	18	18	
1.00-1.19	1		8	11	4	8	1	2	1	4	20	19	1
1.20-1.39		1	8	10	3	5	2	1	1	7	19	13	6
1.40-1.59			1	2		1				2	3	1	2
1.60-1.79													
1.80-1.99													
2.00-2.19													
Total acid- formers.....	4	2	42	34	16	22	11	11	4	18	82	73	9
Mode.....	....	....	0.70	1.10	0.70	1.10	0.90	0.70	0.90	1.30	1.10	1.10	1.30
Mean.....	....	....	0.95	1.08	0.94	1.03	0.97	0.85	1.05	1.11	1.00	0.96	1.32
Probable error			±.17	±.17	±.15	±.17	±.12	±.15	±.11	±.19	±.17	±.16	±.08
Standard devi- ation.....			±.25	±.25	±.22	±.25	±.18	±.22	±.17	±.28	±.26	±.24	±.11
Coefficient of variation....	....	....	26.3	22.9	23.4	24.3	18.6	25.9	16.2	25.2	26.0	25.5	8.3

All the strains considered, somewhat more acid is formed from raffinose (1% normal) than from sucrose (0.7%). No distinct mode

is present, and the dispersion of the distribution is very great, as indicated by a large coefficient of variability (26%).

MacConkey Types I and II generally do not attack raffinose. The few strains in these types which do, are not sufficient for comparative purposes. Type IV tends to form more acid than Type III, but the difference is not considered a reliable index for differentiation.

There is no apparent relation between the animal source and the amount of acid formed from raffinose. The mean for the sewage strains, 1.11% normal acid, is higher than for those from other sources. This difference, as was observed in the case of sucrose, is due to the presence of the Voges-Proskauer-positive group among the sewage strains. The mean for the Voges-Proskauer-negative strains in sewage is 0.94% normal acid.

The Voges-Proskauer-negative strains attack raffinose less readily than do the Voges-Proskauer-positive strains. The means for the two groups are 0.96 and 1.32% normal acid respectively, but the variability among the strains is such as to make the difference (0.36) of questionable significance.

#### C. GLYCEROL

The alcohol, glycerol, is attacked by many strains which form acid but not gas. For calculating acid-production all strains which form more than 0.4% normal acid in 72 hours at 37 C. are regarded as acid-formers. The frequency distributions and the relation of quantitative acid-production to the MacConkey types, to the source, and to the Voges-Proskauer reaction are shown in Table 8. A sharp mode is observed at 0.70% and the mean for all the strains is at 0.73% normal acid.

The MacConkey types are indistinguishable on the basis of quantitative acid-production in 1% glycerol peptone solution altho Type III tends to form somewhat less acid than the others.

The differences between the means of organisms from the various animal sources cannot be regarded as significant. The sheep strains form the least amount of acid (0.60% normal), while the means for strains from other sources are, horse 0.67%, cow 0.71%, man 0.71%, and pig 0.76% normal acid. The somewhat higher mean of the sewage strains (0.83%) is due, again, to the influence of Voges-Proskauer-positive strains. These eliminated, the mean for the Voges-Proskauer-negative strains in sewage is 0.70% normal.

One of the Voges-Proskauer-positive strains does not attack glycerol. This is probably *B. cloacae*. Those Voges-Proskauer-positive organisms which do ferment glycerol, generally form much more acid than the Voges-Proskauer-negative fermenting strains. The mean for the Voges-Proskauer-negative organisms coincides with the mode at 0.70% normal. The Voges-Proskauer-positive organisms have a mode at 1.50%, with a mean at 1.28% normal acid.

TABLE 8  
RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION  
IN GLYCEROL

Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges- Proskauer	
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Neg- ative	Posi- tive
0.00-0.19		1	1	2	1					3	4	3	1
0.20-0.39	2		2	1		2		2		1	5	5	
0.40-0.59	10	1	20	8	8	10	2	4	7	8	39	39	
0.60-0.79	25	16	13	12	5	9	15	13	13	11	66	66	
0.80-0.99	16	8	3	6	5		3	12	3	10	33	31	2
1.00-1.19	1			1					1	1	2	1	1
1.20-1.39				2					1	1	2	1	1
1.40-1.59			3	1						4	4		4
1.60-1.79													
1.80-1.99													
2.00-2.19													
Total acid- formers.....	52	25	39	30	18	19	20	29	25	35	146	138	8
Mode.....	0.70	0.70	0.50	0.70	0.50	0.50	0.70	0.70	0.70	0.70	0.70	0.70	1.50
Mean.....	0.73	0.76	0.67	0.77	0.67	0.60	0.71	0.76	0.71	0.83	0.73	0.70	1.28
Probable error	±.10	±.07	±.17	±.17	±.11	±.06	±.07	±.09	±.13	±.20	±.14	±.11	±.16
Standard devi- ation.....	±.15	±.10	±.25	±.25	±.17	±.09	±.10	±.14	±.19	±.30	±.21	±.16	±.24
Coefficient of variation....	20.5	13.2	37.4	32.5	25.4	15.0	14.1	18.4	26.8	36.2	28.8	24.3	16.0

#### D. DULCITOL

In Table 9 is indicated the relation of the MacConkey types, the source, and the Voges-Proskauer reaction to acid-production in dulcitol.

MacConkey Types I and IV do not form acid from dulcitol. Types II and III produce about equal quantities, 0.88% and 0.81% normal acid respectively, but Type II shows a greater variability.

The sheep, pig, human, and horse strains attack dulcitol more vigorously than do the organisms from the cow. The averages are,

TABLE 9

RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION IN DULCITOL

Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges-Proskauer	
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Negative	Positive
0.00-0.19	53			33	6	11	9	18	19	23	86	80	6
0.20-0.39		1	1	1	2		1				3	3	
0.40-0.59	1	5				1	2		1	2	6	6	
0.60-0.79		7	16		5	1	5	4	2	6	23	23	
0.80-0.99		6	21		6	9	3	4	1	4	27	27	
1.00-1.19		6	1					5	2		7	7	
1.20-1.39			3							3	3		3
1.40-1.59													
1.60-1.79													
1.80-1.99													
2.00-2.19													
Total acid-formers.....	1	24	41		11	11	10	13	6	15	66	63	3
Mode.....	....	0.70	0.90		0.90	0.90	0.70	1.10		0.70	0.90	0.90	1.30
Mean.....	....	0.81	0.88		0.81	0.84	0.72	0.92	0.83	0.85	0.83	0.81	1.30
Probable error		±.15	±.13		±.07	±.08	±.09	±.11	±.15	±.18	±.15	±.11	
Standard deviation.....		±.22	±.20		±.10	±.12	±.14	±.16	±.22	±.26	±.22	±.17	
Coefficient of variation....	....	27.2	22.7		12.3	14.3	19.4	17.4	26.5	30.6	26.5	21.0	

pig 0.92%, sheep 0.84%, human 0.83%, and horse 0.81%, as compared with 0.72% normal acid for cow. The number of fermenting strains from the different sources is too small for reliable comparison and the differences here indicated are insignificant.

Only 3 of the Voges-Proskauer-positive strains ferment dulcitol, but the amount of acid produced by each of these three organisms is greater than that formed by any of the Voges-Proskauer-negative strains. The mean for the Voges-Proskauer-positive organisms is 1.30%, and for the Voges-Proskauer-negative cultures 0.81% normal acid.

E. SALICIN

Acid-production in salicin, as in glycerol, is not always accompanied by gas-formation. The frequency distribution with respect to source, MacConkey type, and Voges-Proskauer reaction is indicated in Table 10.

TABLE 10

RELATION OF SOURCE, MACCONKEY TYPE, AND VOGES-PROSKAUER REACTION TO ACID-PRODUCTION IN SALICIN

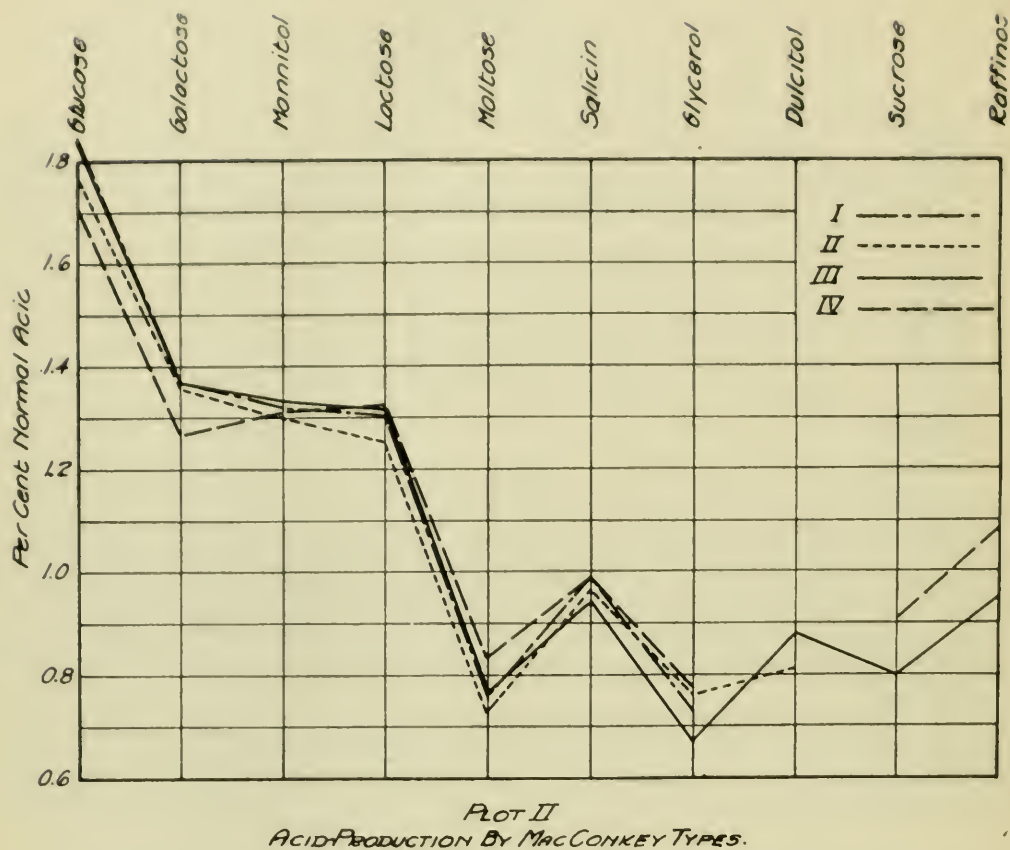
Percentage of Normal Acid	Frequencies												
	MacConkey Type				Source						All Strains	Voges-Proskauer	
	I	II	III	IV	Horse	Sheep	Cow	Pig	Man	Sewage		Negative	Positive
0.00-0.19	26	1	8	8	6	7	1	8	13	8	43	43	
0.20-0.39				1			1				1	1	
0.40-0.59	1			3				2	1	1	4	3	1
0.60-0.79	6	5	10	4	3	6	3	5	4	4	25	25	
0.80-0.99	8	9	16	7	5	6	10	7	5	7	40	40	
1.00-1.19	7	9	4	5	2	3	3	7	2	8	25	23	2
1.20-1.39	6	2	1	4	3		2	2		6	13	11	2
1.40-1.59				1						3	3		3
1.60-1.79			2	1						1	1	1	1
1.80-1.99			1							1	1	1	
2.00-2.19													
Total acid-formers.....	28	25	34	25	13	15	18	23	12	31	112	103	9
Mode.....	0.90	1.00	0.90	0.90	0.90	0.90	0.90	1.00	0.90	1.10	0.90	0.90	1.50
Mean.....	0.98	0.96	0.94	0.98	0.98	0.94	0.94	0.92	0.83	1.11	0.96	0.94	1.28
Probable error	±.16	±.12	±.17	±.21	±.15	±.07	±.12	±.15	±.12	±.21	±.16	±.15	±.22
Standard deviation.....	±.23	±.17	±.26	±.30	±.22	±.11	±.17	±.22	±.17	±.31	±.23	±.22	±.33
Coefficient of variation....	23.5	17.7	27.7	30.6	22.5	11.7	18.1	23.9	20.5	27.9	23.9	23.4	25.8

Inspection of Table 10 shows that the MacConkey types cannot be differentiated on the basis of the amount of acid formed from salicin. Neither is quantitative acid-production an index to the animal source. The Voges-Proskauer-positive strains give more acid (1.28% normal) than the Voges-Proskauer-negative strains (0.94% normal), but the difference is not as marked or distinct as with sucrose and should not be regarded as a differential index.

## RÉSUMÉ

A study of the quantities of acid formed by *Bacillus-coli*-like organisms from different sources (pig, cow, sheep, horse, man, and sewage) when they are inoculated into peptone water containing 1% of various fermentable substances, indicates the following:

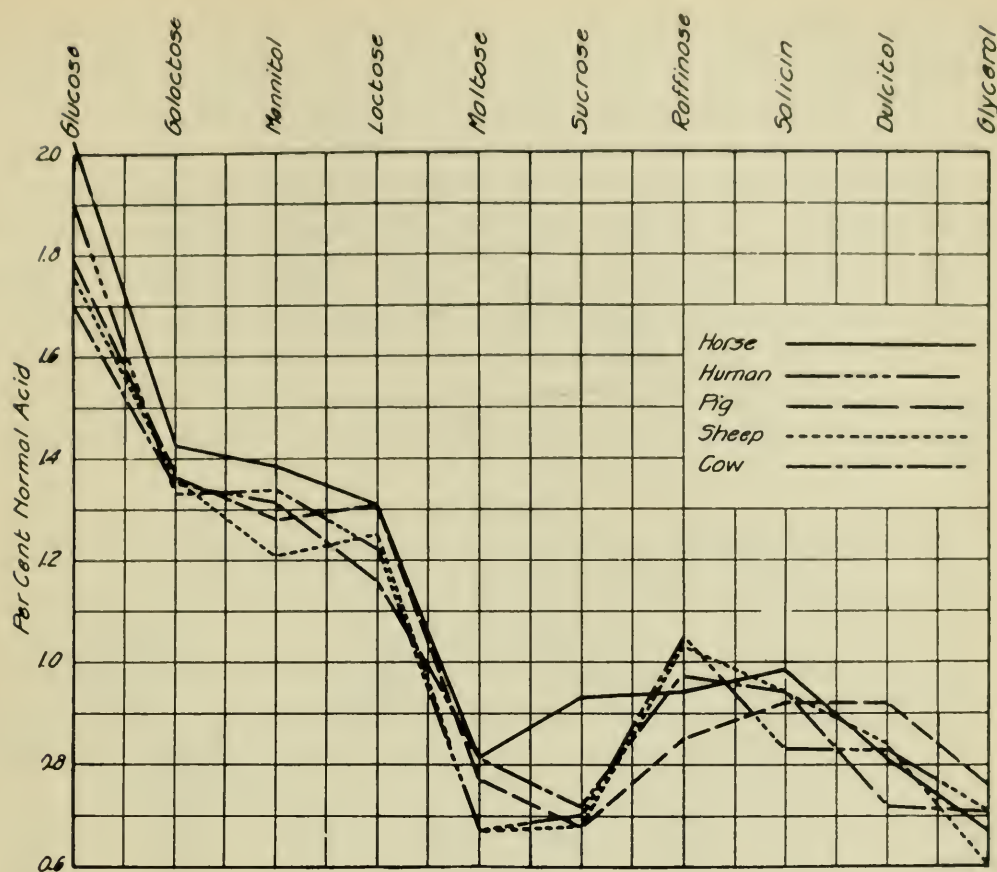
1. The MacConkey types are indistinguishable on the basis of quantitative acid-production in the fermentable carbohydrates, the alcohols, and the glucosid studied. This is shown in Plot II, in which



the curves for the different types run almost parallel and very close together.

2. That there is no correlation between the amount of acid formed from the carbohydrates, the alcohols, and the glucosid studied, and the animal source, is apparent from Plot III, in which a parallelism similar to that in Plot II is observed. The high average of acid-formation in sucrose among the horse strains is due to the presence among them of a few high-acid-producing cultures, not to any ability of horse strains, as a whole, to yield more acid from sucrose. This has been previously indicated in Table 6.

3. In a general way, the Voges-Proskauer-positive strains isolated from sewage form less acid from the monosaccharids, but more acid from the more complex carbohydrates, etc., (except lactose) than the Voges-Proskauer-negative strains. That this difference is not peculiar to the strains isolated for this study, but is characteristic of the Voges-Proskauer-positive and -negative strains in general, is indicated by the



Plot III  
ACID-PRODUCTION BY COLI-LIKE BACTERIA FROM ANIMAL FECES

similar results obtained with the 11 cultures from the collection of the American Museum of Natural History. Four of the museum strains were positive and 7 negative for the Voges-Proskauer reaction.

The average quantities of acid formed from different substances by the Voges-Proskauer-positive and -negative strains obtained from the museum collection and isolated in this laboratory are shown in Table 11 and Plot IV.

The museum strains, both Voges-Proskauer-positive and -negative, form less acid from lactose than the organisms freshly isolated from animals and sewage, but in all other substances tested the differences between the museum and freshly isolated strains are inappreciable. To infer that the museum strains have lost their power to ferment lactose does not offer an adequate explanation of all the phenomena, for it becomes necessary to explain why the organisms should single out and taboo lactose while retaining their power to form acid from the simpler and more easily attacked monosaccharids, as well as the more

difficultly fermented disaccharids, trisaccharid, alcohols, and glucosid studied. No attempt will therefore be made to explain this phenomenon with lactose, except to suggest that it may possibly be attributed to the small number of museum strains studied.

An inspection of Plot IV and Table 11 indicates that all the 167 strains studied considered, the Voges-Proskauer-positive organisms form less acid from glucose than do the Voges-Proskauer-negative strains, and about equal quantities from galactose, mannitol, and lactose. In all other test substances—maltose, salicin, raffinose, dulcitol, glycerol, and sucrose—the Voges-Proskauer-positive strains give rise to more acid, the excess increasing in the order named. The differ-

TABLE 11  
ACID-PRODUCTION IN FERMENTABLE SUBSTANCES BY VOGES-PROSKAUER-POSITIVE AND -NEGATIVE  
BACILLUS-COLI-LIKE BACTERIA

Test Substance	Percentage of Normal Acid				Excess of Acid (in Percentage of Normal) by the V. P. + Organisms	
	American-Museum Strains		Levine's Strains		American- Museum Strains	Levine's Strains
	V. P. —	V. P. +	V. P. —	V. P. +		
Glucose.....	1.82	1.52	1.82	1.46	— .30	— .36
Galactose.....	1.31	1.28	1.36	1.21	— .08	— .15
Lactose.....	0.96	0.85	1.31	1.26	— .11	— .05
Mannitol.....	1.37	1.41	1.31	1.48	+ .04	+ .17
Maltose.....	0.66	1.01	0.75	1.12	+ .35	+ .37
Sucrose.....	0.71	1.52	0.74	1.57	+ .81	+ .83
Raffinose.....	0.79	1.22	0.96	1.32	+ .43	+ .36
Glycerol.....	0.58	1.27	0.70	1.28	+ .69	+ .58
Dulcitol.....	0.83	1.15	0.81	1.30	+ .32	+ .49
Salicin.....	1.00	1.38	0.94	1.28	+ .38	+ .34

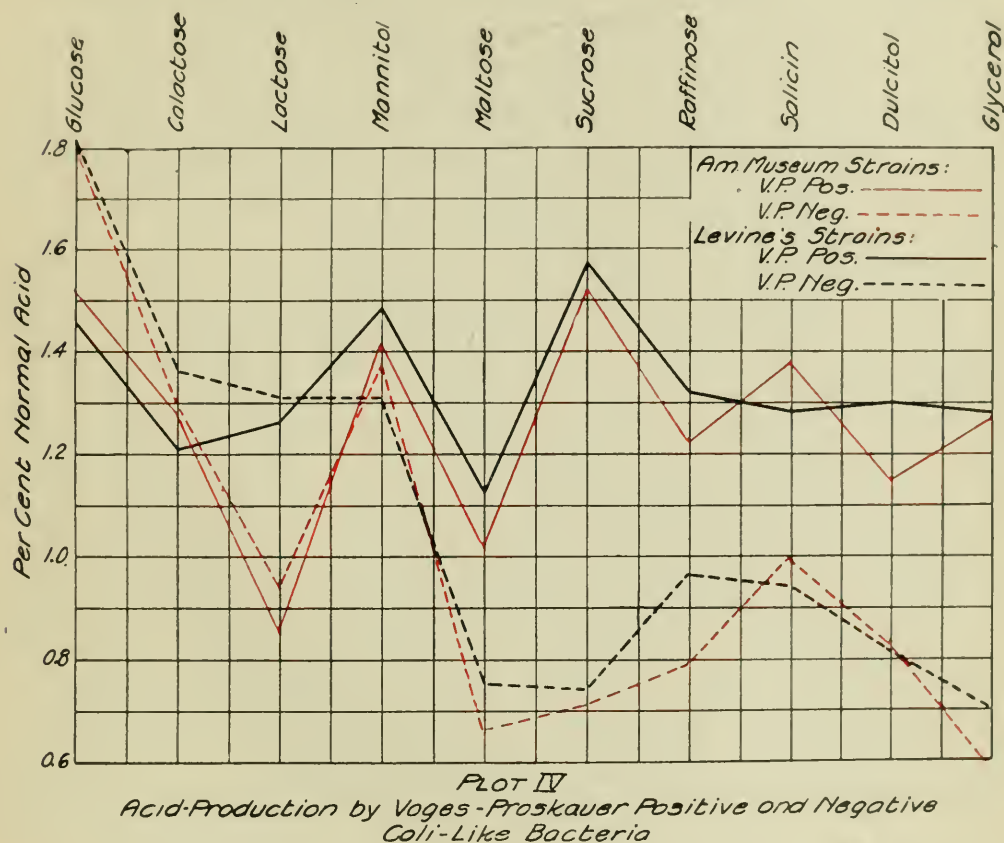
ences obtained in salicin, raffinose, and possibly glucose, are probably not significant, on account of the variations observed in acid-production from these substances.

THE SUPPOSED LOSS OF FERMENTING POWER BY B. COLI IN ITS  
PASSAGE THROUGH SEWAGE

Browne observed that colon-bacillus-like organisms from oysters formed less acid from glucose than did similar organisms derived from man. He concludes: "The bacillus coli isolated from feces, both from laboratory assistants and from the immigrants of the *S. S. Roma*, produced more acid in dextrose and lactose broth than the colon bacillus isolated from oysters. This seems to indicate that *Bacillus coli* loses some of its ability to ferment carbohydrate with the production

of acid during the journey from the intestinal tract to the oysters." He states, however, that in his laboratory experiments he was unable to cause a reduction in fermenting power even after long periods (8 weeks) of storage in sea water.

It appears from this study that a very plausible explanation of Browne's results is that Voges-Proskauer-positive organisms were among his oyster strains. Such organisms are very rare in feces, but not uncommon in sewage and soil washings. The admixture of a



few Voges-Proskauer-positive organisms in a collection of colon-bacillus-like strains would decrease the mean amount of acid formed from glucose and raise the titer of that from sucrose. The oyster strains employed by Browne formed less acid from glucose, and somewhat more from sucrose, than the fecal strains, thus confirming to some extent the inference that the differences he observed were due to an admixture of a few Voges-Proskauer-positive organisms rather than to a loss of fermenting power by colon-bacillus-like strains in their passage through sewage.

THE SUBSTITUTION OF QUANTITATIVE ACID-PRODUCTION FOR GAS-  
FORMATION AS A DIFFERENTIAL INDEX IN STUDIES  
ON *B. COLI*

Kligler<sup>6</sup> suggests that quantitative acid-production be substituted for gas-formation as an index of fermentation. He points out that in standard meat-infusion sugar-freed carbohydrate broth media there is a rather sharp dividing line between acid-producers and nonacid-producers at 1.5% normal acid, and that quantitative gas-production is variable and unreliable. Of course it is agreed that, as a quantitative test, gas-formation as ordinarily determined in the Smith or Durham tube is of little value; as a qualitative test, however, it may be of considerable significance. If a culture is inoculated into sugar broth and gas is formed, while no gas is produced in plain broth, the organism would most certainly be regarded as a fermenter irrespective of whether more or less than 1.5% acid is formed.

Kligler apparently regards such an organism as a nonfermenter, for he says: "The members of the proteus group, on the other hand, produced from 10 to 20 per cent. gas in lactose broth tho at no time did they produce more than 1.0 percent normal acid," and he later records this group as lactose-negative. It is not the intention to debate at this point whether *B. proteus* is a lactose-fermenter or not, but it should be pointed out that to say that an organism which forms gas from a carbohydrate is a nonfermenter because the acid titer is low, introduces confusion into the already much maligned and abused term "fermentation." The low titer might be due to a secondary alkali-production which masks the acid, as suggested by Rogers. It has been repeatedly observed in this laboratory that *B. aerogenes* in peptone dipotassium-phosphate solution containing 1 or 2% glucose, may be acid to methyl red after 24 hours' incubation, but alkaline after from 48 to 96 hours at 37 C.

Rogers, Clark, and Evans<sup>7</sup> also determined titratable acid and selected 1% normal acid as the point of demarcation between fermenters and nonfermenters, but they point out the possible errors in acid-determination and give precedence to gas-formation as indicated in the following:

"Under certain circumstances which have not yet been definitely determined, the acid from the fermentation of sugar may be masked by a secondary alkaline production, sufficient in some cases entirely to obscure the acid for-

<sup>6</sup> Jour. Infect. Dis., 1914, 15, p. 137.

<sup>7</sup> (a) Jour. Infect. Dis., 1914, 14, p. 411; (b) 15, p. 100; (c) 1915, 17, p. 137.

mation. In one small group of this collection, the lactose broth tubes at the end of seven days were only slightly more acid than the blank, altho all of the cultures gave gas in lactose bile. In no case was the titration of the culture less than that of the blank, altho this was usually the case with broths in which there was no fermentation. Where positive evidence of the fermentation of a sugar was obtained in another way, the negative evidence of the titration was disregarded and in the correlation tables the culture was included with the positive reactions. If, for instance, the titration of lactose broth was negative, while the lactose bile fermentation tubes showed gas, the cultures were considered to be lactose positive."

Acid-production should not be given precedence over gas-formation. They may be independent characters. If however, after careful studies, it appears that there is a marked correlation between quantitative acid-production and qualitative gas-formation, then it may be feasible to supplement, if not substitute, the gas test by the acid test. In that event, the line of demarcation between fermenters and non-fermenters would have to be determined for the medium employed. In this study, with peptone water containing 1% carbohydrate, non-fermenters rarely produced as much as 0.2% normal acid.

Another point of disagreement as to acid-production by *B. coli* is the maximal amount of acid formed. Kligler,<sup>6</sup> using meat-infusion media, often obtained titers of 4% normal acid or more, and similar results have been recorded by Rogers.<sup>7</sup> Browne,<sup>1</sup> however, using Liebig's meat-extract media, states that the limiting acidity for *B. coli* is 2.4% normal acid as determined by titration with phenolphthalein. Winslow and Walker<sup>8</sup> determined the acid-production in 12 substances by *B. coli*. The maximal acidity observed was 0.45 c.c. N/20 NaOH to the cubic centimeter of culture medium, or 2.25% normal acid.

In the study recorded here, with peptone water as the basic medium, the results are in entire accord with Winslow and Walker's, and with Browne's. Of more than 2500 titrations, none showed more than 2.4% normal acid.

The difference in acid-production observed by various investigators is probably due to differences in the composition of the media employed. It is now well established that more acid is formed in meat-infusion broth than in beef-extract broth. In media containing much phosphates, as yeast water, even more acid is formed than in meat-infusion broth. Within certain limits the amount of acid formed, as determined by titration with phenolphthalein, is a function of the amount of buffer substances (as  $K_2HPO_4$  amino-acids, extractives, etc.) present in the culture medium. Acid is formed until a certain

<sup>8</sup> Science, 1907, 26, p. 797.

H<sup>+</sup>-ion concentration is reached. The ratio of the total titratable acid formed to the maximal or limiting H<sup>+</sup>-ion concentration is not constant, but varies, within limits, with the amount of buffer materials present in the medium.

The limiting H<sup>+</sup>-ion concentration may be an index of (1) the resistance of an organism to acid (H<sup>+</sup> ions), or (2) the point of equilibrium between the decomposing carbohydrate and its end products under the influence of an organism.

If the limiting H<sup>+</sup>-ion concentration in glucose broth is such as to inhibit further growth of the organism, then the organism will die and the H<sup>+</sup>-ion concentration will remain constant. This seems to be the course of events with the Voges-Proskauer-negative group. With the Voges-Proskauer-positive organisms the H<sup>+</sup>-ion concentration rises to a maximum and then decreases, the medium becoming alkaline to methyl red. Under these conditions it is inferred that the maximal H<sup>+</sup>-ion concentration is a measure of the point of equilibrium between glucose and its end products under the influence of the organism in question.

It may be further considered that after the limiting H<sup>+</sup>-ion concentration is reached, the organism, if not destroyed, will, if capable, attack the peptones forming alkali. Some of the free acid becomes neutralized and more carbohydrate may be decomposed. The H<sup>+</sup>-ion concentration would remain constant as long as there is any fermentable carbohydrate present. If this assumption is correct, then an increase of the carbohydrate should retard the reversion from an acid to an alkaline reaction. This is exactly what takes place. In some work now in progress it has been found that Voges-Proskauer-positive strains were alkaline to methyl red after 24 hours' incubation in 0.5% peptone dipotassium-phosphate solution containing 0.5% glucose. In the same medium with 1% glucose, the reaction was acid after 24 hours but alkaline after from 48 to 72 hours. With 2% glucose, the acid reaction persisted until the 4th or 5th day. With 5% glucose there was no reversion to an alkaline reaction even after several weeks.

#### THE CORRELATION OF ACID- AND GAS-FORMATION

Table 12 shows the relation of gas-production to the amount of acid formed from sucrose, raffinose, dulcitol, glycerol, and salicin. The other test substances are not indicated because they were invariably fermented with production of gas. Cultures are regarded as gas-

formers if gas is observed in the closed arm irrespective of the quantity.

Table 12 indicates that with sucrose, raffinose, and dulcitol, acid- and gas-production after 36 hours' incubation at 37 C. are strikingly correlated. Of 80 organisms which fail to form gas from sucrose, 79 (98.8%) form less than 0.2% normal acid, and the remaining culture forms only 0.2% normal acid. Among the 75 organisms which do give gas from sucrose, 8 (10.6%) form more than 0.4 but less than 0.6%, 48 (64%) give between 0.6 and 0.79%, and the remaining 19 strains (25.4%) form more than 0.8% normal acid. There is no overlapping whatever between the amounts of acid produced by the gas-formers and the nongas-formers. To summarize: of the 80 strains which fail to produce gas, none form more than 0.2% normal acid, while among the 74 gas-producers the minimal amount of acid produced in peptone solution containing 1% sucrose is more than 0.4% normal acid.

A similar correlation is observed between acid- and gas-formation in 1% dulcitol in peptone solution. Of 88 strains that do not form gas, 86 (97.8%) give less than 0.2% normal acid. The remaining two organisms form 0.3% and 0.4% normal acid. Among the 67 gas-formers, however, there are only 2 (3%) that produce less than 0.4% normal acid.

The correlation of acid- and gas-production in peptone raffinose solution is also very marked; 79 produce gas and 77 fail to form gas from raffinose. Of the nongas-formers 72 (93.5%) form less than 0.2% normal acid; 3 organisms (3.9%) between 0.2% and 0.6% acid; and 2 cultures (2.6%) more than 0.8% acid. Among the gas formers 1 culture (1.3%) produces no acid, while 2 others (2.5%) form less than 0.6% normal acid. The other 76 gas-formers (96.2%) form more than 0.6% normal acid.

With glycerol and salicin the correlation of acid-production and gas-formation is not nearly so striking as it is with sucrose, dulcitol, or raffinose.

Gas is formed from glycerol by 118 of the cultures after 72 hours' incubation, while 38 organisms do not form gas. Of the gas-formers, 16 (13.6%) produce 0.4-0.59% normal acid as compared with 23 (60.6%) of the nongas-formers, while 61 (51.7%) of the former and 5 (13.2%) of the latter give 0.6-0.79% normal acid. One organism which does not form gas yields more than 0.8% normal acid.

TABLE 12

RELATIONSHIP BETWEEN QUANTITATIVE ACID-PRODUCTION AND GAS-FORMATION BY B. COLI

Test Substance	Gas	Percentage or Normal Acid					
			0-0.19	0.20-0.39	0.40-0.59	0.60-0.79	0.80 or more
Sucrose.....	{ +	{ No.	0	0	8	48	19
		{ %			10.6	64.0	25.4
	{ -	{ No.	79	1	0	0	0
		{ %	98.8	1.2			
Raffinose.....	{ +	{ No.	1	0	2	18	58
		{ %	1.3		2.5	22.8	73.4
	{ -	{ No.	72	1	2	0	2
		{ %	93.5	1.3	2.6		2.6
Dulcitol.....	{ +	{ No.	0	2	5	23	37
		{ %		3.0	7.5	34.3	55.2
	{ -	{ No.	86	1	1	0	0
		{ %	97.8	1.1	1.1		
Salicin.....	{ +	{ No.	0	0	1	19	82
		{ %			10	18.6	80.4
	{ -	{ No.	43	1	3	6	1
		{ %	79.7	1.8	5.6	11.1	1.8
Glycerol.....	{ +	{ No.	0	0	16	61	41
		{ %			13.6	51.7	34.7
	{ -	{ No.	4	5	23	5	1
		{ %	10.5	13.2	60.5	13.2	2.6

Salicin is fermented, with gas-formation, by 102 organisms after 72 hours at 37 C., while 54 strains do not form gas. Among the non-gas-formers, 10 (18.5%) produce 0.4-0.8% normal acid, whereas this quantity of acid is also formed by 20 (19.6%) of the gas-formers.

It appears from Table 12 that under the conditions of these experiments, acid-production in sucrose, dulcitol, and raffinose is well correlated with the presence or absence of gas. With salicin the correlation is not so marked, while with glycerol the line of demarcation between gas-formers and nongas-formers, as indicated by the quantity of acid produced, is very indistinct. The substitution of quantitative acid-production for gas-formation would therefore be particularly undesirable when working with glycerol.

These results are well in accord with those of Winslow and Walker,<sup>8</sup> who observe: "Gas-formation coincided with acidity except in the case of dextrin." Unfortunately, acid-formation in dextrin was not determined in this study, and Winslow and Walker did not employ salicin or glycerol.

#### CHARACTERISTICS OF ORGANISMS FROM THE DIFFERENT SOURCES

When this study was begun (1915), motility and fermentation of dextrin and starch were regarded as of little significance and hence these tests were omitted. In the following year (1916) the possible significance of the reactions was realized and, as the cultures were still available, they were tested out. Motility was determined in a soft agar medium consisting of nutrient broth and 0.5% agar.

In Table 13 are shown the number and percentage of organisms giving positive reactions with the various tests. Glucose, galactose, mannitol, maltose, and lactose are fermented by all strains, with gas-production. Inulin is not fermented by any of the organisms, and gelatin is uniformly negative in 20 days at 20 C. Gas is formed from glycerol by 76.2%, from salicin by 66.1%, from raffinose by 50.7%, from sucrose by 48.7%, from dulcitol by 43.6%, from dextrin by 5.1%, and from starch by 4.5%. The Voges-Proskauer reaction is given by 5.8%, indol is produced by 91.1%, and 61.5% are motile.

Table 14 shows the characters of organisms isolated from different sources. Characters which are negative or positive for all strains are omitted.

Several things are evident. Organisms giving a positive Voges-Proskauer reaction or gas from dextrin and starch were obtained

TABLE 13

GAS-FORMATION AND OTHER CHARACTERISTICS OF *BACILLUS-COLI*-LIKE BACTERIA FROM VARIOUS ANIMALS AND SEWAGE

Character	Number Positive	Percentage Positive
Motility.....	96	61.5
Gelatin.....	0	0
Indol.....	142	91.1
Voges-Proskauer.....	9	5.8
Glucose.....	156	100
Galactose.....	156	100
Mannitol.....	156	100
Dulcitol.....	68	43.6
Glycerol.....	118	76.2
Maltose.....	156	100
Lactose.....	156	100
Sucrose.....	76	48.7
Raffinose.....	79	50.7
Salicin.....	102	66.1
Dextrin.....	8	5.1
Inulin.....	0	0
Starch.....	7	4.5

only from sewage. This must not be taken to mean that such organisms are entirely absent from the other sources, but it certainly indicates that they are extremely scarce in feces of the animals studied.

Salicin is fermented by 95% of the bovine strains, and by 8 (89%) of the 9 Voges-Proskauer-positive strains from sewage. Organisms

TABLE 14

MOTILITY AND OTHER REACTIONS OF *BACILLUS-COLI*-LIKE BACTERIA FROM DIFFERENT SOURCES

Source	Horse	Sheep	Cow	Pig	Man	Sewage	
						V. P. —	V. P. +
Number of strains.....	19	22	20	31	25	30	9
Percentage of Positive Reactions							
Motility.....	100.0	77.3	80.0	93.7	32.0	20.0	11.1
Voges-Proskauer reaction....	0.0	0.0	0.0	0.0	0.0	0.0	100.0
Indol.....	100.0	100.0	100.0	93.7	84.0	83.5	66.7
Sucrose.....	79.0	95.5	50.0	32.3	12.0	26.6	100.0
Raffinose.....	73.8	100.0	50.0	32.3	16.0	33.3	100.0
Dulcitol.....	68.5	50.0	50.0	42.0	20.0	43.3	33.3
Glycerol.....	84.3	62.0	95.0	74.2	64.0	70.0	88.9
Salicin.....	73.8	68.3	95.0	58.1	44.0	60.0	88.9
Dextrin.....	0.0	0.0	0.0	0.0	0.0	0.0	88.9
Starch.....	0.0	0.0	0.0	0.0	0.0	0.0	77.8

from other sources attack salicin less readily—horse 73.8%, sheep 68.3%, pig 58.1%, man 44%, and sewage (Voges-Proskauer-negative strains) 56.7%. This glucosid was used by MacConkey,<sup>3</sup> who did not regard its employment worth while for classification purposes. Recently (1914) Kligler<sup>6</sup> suggested that salicin displace dulcitol in

subdivision of the colon-bacillus group, but Rogers<sup>7</sup> questions the value of salicin in view of the very large number of his strains which attacked it. In this connection it might be well to point out that organisms studied by Rogers consisted of bovine strains, grain strains (probably Voges-Proskauer-positive organisms), and milk strains (which may be considered for the most part as a mixture of bovine and grain strains). In view of the results obtained here with bovine and Voges-Proskauer-positive strains, and by Kligler with Voges-Proskauer-positive strains, it would be expected that more than 90% of Rogers' cultures would attack salicin. It appears then that salicin-fermentation is somewhat correlated with the source.

Glycerol is also fermented by almost all the Voges-Proskauer-positive and bovine strains and less frequently by organisms from the other animals, but the difference is less marked than with salicin.

Dulcitol is only occasionally fermented by the human and Voges-Proskauer-positive strains, but there seems to be very little relation between dulcitol-fermentation and the animal source.

Indol-production is not correlated with the animal source.

In motility there is a marked contrast between the strains from horse, sheep, cow, and pig on the one hand, and those from man and sewage on the other. Less than one-third of the sewage and human strains are motile, as compared with more than four-fifths of the other animal strains. McWeeney<sup>9</sup> found nonmotile *B. coli* abundant in feces, and notes that Stocklin also had observed many nonmotile forms among fecal strains. Just what significance is to be attached to motility is hard to say at present, because so few bacteriologists determine this character in routine work. MacConkey, however, strongly advocates the test. As determined in the 0.5% agar medium the motility test is simple, quick, and not at all burdensome.

Sucrose and raffinose are so well correlated that a consideration of either will suffice for both. The Voges-Proskauer-positive and sheep strains are practically all sucrose-fermenters (100% and 95.5% respectively). Of the horse strains 79%, and of the organisms from the cow 50% form gas from sucrose; only 32.3% of strains from the pig, 26.6% of those from sewage (Voges-Proskauer-negative strains), and 12% of those from man form gas from sucrose. That such a small number of human strains attack sucrose is particularly interesting, and a review of the literature indicates that similar results have

<sup>9</sup> Cited by Prescott and Winslow, *Elements of Water Bacteriology*, 1913.

TABLE 15

FERMENTATION OF SUCROSE BY *BACILLUS-COLI*-LIKE BACTERIA FROM HUMAN FECES

Investigators	Number of Organisms Studied	Number of Sucrose Fermenters	Percentage of Sucrose Fermenters
Houston, <sup>11</sup> 1902-3.....	100	30	30
MacConkey, <sup>3</sup> 1905 and 1909.....	419	142	33.9
Ferreira, <sup>12</sup> Horta, Paredes, 1908.....	117	44	37.6
Winslow <sup>8</sup> and Walker, 1907.....	25	8	32
Howe, <sup>10</sup> 1912.....	540	324	60
Clemesha, <sup>13</sup> 1912.....	1200	348	29
Browne, <sup>1</sup> 1915.....	175	20	11.3
Levine, <sup>4</sup> 1916.....	25	3	12
Total.....	2601	919	35.3

been obtained by previous investigators. In Table 15 is shown the proportion of sucrose-fermenters obtained from human feces by different investigators. Howe<sup>10</sup> found 60% of 540 *Bacillus-coli*-like organisms to be sucrose fermenters, but the other investigators usually found twice as many nonfermenters as fermenters. Of 2601 cultures of human colon bacilli studied by various observers, at different times and in different countries, only 35.3% fermented sucrose.

In connection with the study reported here it should be noted that the number of human strains isolated is small, and that they were collected in the winter. Clemesha,<sup>13</sup> and also Browne,<sup>1</sup> call attention to "epidemics" of certain types of *B. coli*, and to seasonal variations. These phases need further investigation.

#### CONCLUSIONS

In studies on quantitative acid-production the average should be supplemented with a statement of its deviation measures; the unqualified average may lead to a misconception of the acid-producing properties of a group of organisms.

Quantitative acid-production in glucose, galactose, maltose, lactose, sucrose, raffinose, salicin, inulin, mannitol, dulcitol, and glycerol, is not a reliable index for differentiating colon-bacillus-like bacteria derived from pig, horse, sheep, cow, or man.

The MacConkey types are indistinguishable on the basis of quantitative acid-production in the fermentable carbohydrates, the alcohols, and the glucosid studied.

<sup>10</sup> Science, 1912, 35, p. 225.

<sup>11</sup> Suppl. to 32nd Ann. Rep. containing Rep. of Med. Officer for 1902-1903, p. 511.

<sup>12</sup> Arch. d. Real Inst. Bacteriol. Camara Pestana, 1908, 2, p. 153.

<sup>13</sup> Jour. Hyg., 1912, 12, p. 463.

The Voges-Proskauer-positive strains (*aerogenes-cloacae* group) form somewhat less acid from glucose, but more acid from maltose, sucrose, glycerol, and dulcitol, and possibly also from raffinose and salicin, than do the Voges-Proskauer-negative strains (*colon-bacillus* group).

Acid-formation should not be given precedence over gas-formation in studies on *B. coli*, for the acid may be masked by a secondary alkali-production. In general, however, acid-production is accompanied by gas-formation. With sucrose, dulcitol, and raffinose, acid-production and gas-formation are almost perfectly correlated. The correlation is less marked in the case of salicin, while the line of demarcation between gas-formers and nongas-formers, as indicated by quantitative acid-production from glycerol, is very indistinct.

Practically all Voges-Proskauer-positive and bovine strains attack salicin with liberation of gas. This glucosid is fermented less frequently by the organisms from pig, horse, sheep, man, and sewage

Gas is formed from sucrose as follows: Voges-Proskauer-positive (Voges-Proskauer-negative strains).

strains 100%, sheep 95.6%, horse 79%, cow 50%, pig 32.3%, sewage (Voges-Proskauer-negative strains) 26%, and human strains 12%.

Of 2601 human strains of *B. coli* studied by different investigators, in various countries and at different times, only 35.3% have been sucrose-fermenters.

Motility, as determined in semisolid nutrient agar, seems to be an important character. Only 32% of the human and 20% of the Voges-Proskauer-negative sewage strains are motile, as compared with 93.7% of pig, 80% of cow, 77.3% of sheep, and 100% of horse strains.

## RELATIONSHIP BETWEEN SERUM REACTIONS \*

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It has been generally accepted that there is no relation between serum reactions in so far as the numeric values of the titers are concerned; that, in other words, the serologic qualities of an animal are so irregular in their correlations that no conclusion concerning one serum reaction can be arrived at by knowledge of another. However, in the course of routine work with complement-fixation I often have had the impression that rabbit sera which give high hemolytic titers show also high precipitins (the latter being due to small quantities of serum adhering to the sheep corpuscles), and as the question involved is not only of interest but of great importance, I have found it advisable to make systematic tests. Furthermore, one of our chief problems here is to find an easier method of testing the potency of hog-cholera immune serum, a question intimately connected with the relationship of serum reactions.

There are two kinds of reactions which present the greatest probability of a connection: that in which the antigen is uniform tho the antibodies differ, and that in which the antigen is multiform tho the titrated antibodies are of the same kind. To test the first class of reactions I selected a blood-corpuscle emulsion as antigen which injected into an animal produces several different antibodies, 2 of which, agglutinins and hemolysins, are easily titrated.

*Experiment 1.*—Five rabbits were injected intraperitoneally with 5 c.c. of a 5% emulsion of sheep corpuscles each. The injections were repeated on the 5th and 10th days. After 5 days blood was drawn from these animals and the agglutinins and hemolysins titrated. All the agglutinins were titrated after the following formula:

Tube	Salt Solution	Serum	Emulsion	Dilution
1		0.5 c.c. (10%)	0.5 c.c.	1:20
2	0.5 c.c.	0.5 c.c. of Tube 1	0.5 c.c.	1:40
3	0.5 c.c.	0.5 c.c. of Tube 2	0.5 c.c.	1:80
4	0.5 c.c.	0.5 c.c. of Tube 3	0.5 c.c.	1:160
5	0.5 c.c.	0.5 c.c. of Tube 4	0.5 c.c.	1:320
6	0.5 c.c.	0.5 c.c. of Tube 5	0.5 c.c.	1:640
7	0.5 c.c.	0.5 c.c. of Tube 6	0.5 c.c.	1:1280
8	0.5 c.c.	0.5 c.c. of Tube 7	0.5 c.c.	1:2560

\* Received for publication September 19, 1916.

The titration of hemolysins employed 0.5 c.c. serum dilution, 1 c.c. salt solution, 0.5 c.c. complement (5% guinea-pig serum), and 0.5 c.c. blood-corpuscle emulsion (4%). The dilutions of serum used were 1:100, 1:500, 1:1000, 1:2000, 1:3000, 1:5000.

The results of this first experiment were as follows:

Rabbit	Agglutinins	Hemolysins
1.....	1:160	1:1000
2.....	1:320	1:100
3.....	1:160	1:3000
4.....	1:1280	1:100
5.....	1:320	1:500

Apparently there is no relation in strength between the two reactions in these five cases.

Experiment 1 was repeated with 5 other rabbits, one of which died before the blood could be taken.

Rabbit	Agglutinins	Hemolysins
1.....	1:320	1:600
2.....	1:1280	1:800
3.....	1:640	1:2000
4.....	1:640	1:500

Here too the incongruity of the two reactions is apparent.

The same experiment with guinea-pigs gave the following titers:

Guinea-Pig	Agglutinins	Hemolysins
1.....	1:2560	1:2000
2.....	1:640	Below 1:100
3.....	1:2560	1:1000
4.....	1:1280	1:2000
5.....	1:640	1:100

In this case the two lowest agglutinating titers correspond to the two lowest hemolytic titers.

*Experiment 2.*—This experiment, complicated by the simultaneous use of 3 blood emulsions at the same time, belongs partly to the second class of reactions. An emulsion consisting of equal amounts of horse, sheep, and pig corpuscles was used. It was intravenously injected in doses of 2 c.c., and the treatment after 5 days was repeated. The reactions may be compared in view of the first class of reactions as follows:

Rabbit	Agglutinins			Hemolysins		
	Horse Corpuscles	Pig Corpuscles	Sheep Corpuscles	Horse Corpuscles	Pig Corpuscles	Sheep Corpuscles
1	1:5120	1:320	1:320	1:100	Below 1:100	1:1000
2	1:5120	1:640	1:320	1:1000	Below 1:100	1:1000
3	1:2560	1:320	1:640	1:100	Below 1:100	1:5000
4	1:2560	1:160	1:40	Below 1:100	1:100	1:1000
5	1:1280	1:1280	1:320	1:100	Below 1:100	1:3000

These three groups show only a very slight parallelism between the two reactions. The comparison in the sense of the second group can be made directly on the tabulations. The arrangement of the sera according to the numeric values of their titers is as follows:

Agglutinins		
Horse Corpuscles No. 1, No. 2, No. 3, No. 4, No. 5,	Pig Corpuscles No. 5, No. 2, No. 1, No. 3, No. 4.	Sheep Corpuscles No. 3, No. 1, No. 2, No. 5, No. 4.
Hemolysins		
Horse Corpuscles No. 2, No. 1, No. 3, No. 5, No. 4.	Pig Corpuscles No. 4, No. 3, No. 5, No. 1, No. 2.	Sheep Corpuscles No. 3, No. 5, No. 1, No. 2, No. 4.

The results of the comparisons are apparently negative.

Experiment 2 was repeated, intraperitoneal injections of 5 c.c. of mixed emulsion being used, with 5 other rabbits, 2 of which were lost. Below are the titers of the three remaining:

Rabbit	Agglutinins			Hemolysins		
	Horse Corpuscles	Pig Corpuscles	Sheep Corpuscles	Horse Corpuscles	Pig Corpuscles	Sheep Corpuscles
1	1:640	1:160	1:640	1:500	1:100	1:3000
2	1:1280	1:1280	1:320	1:500	1:100	1:1000
3	1:320	1:160	1:40	1:100	1:1000	1:3000

*Experiment 3.*—In this experiment the rabbits of the first experiment were employed. They were injected 3 times at intervals of 5 days with an emulsion of B. paratyphosus B. This was prepared by washing off 24-hour-old agar cultures with 10 c.c. of normal salt solution and heating the emulsion for 45 minutes at 65 C. Seven days after the last injection these rabbit sera were titrated for agglutinins. The results compared with the previously ascertained hemagglutinin titers are as follows:

Rabbit	Agglutinins for B. paraty- phosus B	Agglutinins for Sheep Corpuscles
1.....	1:1280	1:160
2.....	1:5120	1:320
3.....	1:2560	1:160
4.....	1:320	1:1280
5.....	1:1280	1:320

There is no relation between these two titers.

*Experiment 4.*—The agglutinin titers against 3 different antigens were compared. Emulsions of *B. coli*, *B. subtilis*, and *B. pyocyaneus* were used on 5 rabbits. The technic was the same as in Experiment 3, except that the emulsions were counted and their concentrations equalized.

Rabbit	Agglutinins		
	<i>B. Coli</i>	<i>B. Subtilis</i>	<i>B. Pyocyaneus</i>
1.....	1:320	1:80	1:40
2.....	1:160	1:160	Below 1:20
3.....	1:1280	1:640	1:80
4.....	1:320	Below 1:20	1:20
5.....	1:320	1:1280	1:40

Serum 3 excepted, there is hardly any parallelism between the titers. The sera, arranged according to the titration, are found as follows:

For <i>B. coli</i>	For <i>B. subtilis</i>	For <i>B. pyocyaneus</i>
No. 3	No. 5	No. 3
No. 4	No. 3	No. 5
No. 5, No. 1	No. 2	No. 1
No. 2	No. 1	No. 4
	No. 4	No. 2

The repetition of Experiment 4 gave the following results:

Rabbit	Agglutinins		
	<i>B. Coli</i>	<i>B. Subtilis</i>	<i>B. Pyocyaneus</i>
1.....	1:640	1:320	1:80
2.....	1:160	1:160	1:640
3.....	1:640	1:160	1:640

Rabbits 4 and 5 were accidentally lost. The comparison of these tabulations gives a negative result.

*Experiments 5 and 6.*—Seroprecipitins against 3 different antigens were compared. Five rabbits were injected 3 times intravenously at intervals of 5 days. An equal mixture of horse, pig, and sheep serum was used in doses of 6 c.c. For the titration different dilutions of horse, pig, or sheep serum were underlaid with 0.1 c.c. of the rabbit serum to be titrated.

Rabbit	Seroprecipitins		
	Horse	Pig	Sheep
1.....	1:1000	1:1000	1:3000
2.....	Below 1:100	1:100	1:100
3.....	1:100	1:1000	1:1000
4.....	1:1000	1:5000	1:5000
5.....	1:5000	1:5000	1:3000

With the exception of the titer of Rabbit Serum 4 for horse serum, the coincidence of these titers is manifest. Below the sera are arranged according to their titration:

For Horse Serum	For Pig Serum	For Sheep Serum
No. 5	No. 4	No. 4
No. 4, No. 1	No. 5	No. 5
No. 3	No. 3, No. 1	No. 1
No. 2	No. 2	No. 3
		No. 2

This experiment was repeated with the following results:

Rabbit	Seroprecipitins		
	Horse	Pig	Sheep
1.....	1:1000	1:3000	1:5000
2.....	1:500	1:1000	1:1000
3.....	1:500	1:500	1:500
4.....	1:1000	1:1000	1:1000
5.....	1:1000	1:1000	1:3000

In this tabulation too a parallelism of the titer values cannot be denied.

SUMMARY

There was no coincidence between the titers of hemagglutinins and hemolysins against the same antigen.

There was none between the titers of hemagglutinins against different antigens or between the titers of hemolysins against different antigens.

There was none between titers of agglutinins for bacteria, when 3 different antigens were employed, namely, B. coli, B. subtilis, B. pyocyaneus.

In 2 tests, each of 5 rabbits, seroprecipitins were produced rather evenly, 3 different antigens being used.

# THE ETIOLOGY OF TYPHUS EXANTHEMATICUS IN MEXICO (TABARDILLO) \*

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In a preliminary report<sup>1</sup> we presented an outline of the results of our investigations on typhus exanthematicus in Mexico. It is our purpose, in this communication, to state the facts which led us to our conclusions as well as to bring forward other phases of the work not mentioned in the first paper.

The studies here reported were made in Matehuala, in Central Mexico, during the epidemic of typhus fever which prevailed throughout the winter, or dry months, of 1915-1916. An expedition consisting of the authors was made possible through the generosity of the board of directors of Mount Sinai Hospital. A complete laboratory equipment including animals for experimental purposes was taken with us in a special car. We wish to thank Dr. F. S. Mandlebaum, pathologist to the hospital, for his great aid in this direction. These investigations could not have been made without the assistance of the American Smelting and Refining company, who magnanimously offered to take the expedition into Mexico privately and allowed the use of one of their plants as a base for operations.

Matehuala, where we did our work, is a town of 10,000 inhabitants. The extent of the epidemic both in this locality and in the remainder of Mexico was extremely difficult to determine on account of the absence of vital statistics, and the lack of adequate governmental or municipal control of health and sanitation in the district in which we worked. All the available data, however, pointed to a widespread epidemic. From reports of individuals and official newspapers it was estimated that there were 30,000 cases, or 10% of the population, in Mexico City alone. We estimated that there were at least 500 typhus-fever patients in Matehuala during the dry season.

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<sup>1</sup> Jour. Am. Med. Assn., 1916, 66, p. 1692.

It is important to note, when comparing our observations in Mexico with those on endemic typhus fever in New York and on the severe type in Southeastern Europe in 1913 and 1915, that this epidemic, while wide in extent, was but moderate in severity; the mortality in our series of cases was 20%. Arranged in the order of severity, indicated by the rate of mortality, we find:

1. Mildest, the endemic typhus cases in New York (Brill's disease).

2. Next severe, the epidemic typhus cases in Bulgaria and Russia in 1915-1916.

3. Still more severe, the epidemic typhus cases in Mexico.

4. Severest, the epidemic typhus occurring in immigrants from the Balkans in 1914.

The details of the cases included in (1) and (4) were given in the original publication by Plotz, Olitsky, and Baehr;<sup>2</sup> those in (2) will form the subject of a paper, shortly to appear, by Plotz and Baehr; while those mentioned in (3) will be discussed in this communication.

Our work, which was begun Feb. 1, 1916, was interrupted by the unsettled political conditions in Mexico on March 8, 1916. About the same time Dr. Husk became infected with typhus fever and died.

#### VIRUS STUDIES

In its larger features the disease as the authors found it in Matuhuala corresponded to the classical type. Yet it became essential, since influenza, relapsing fever, typhoid fever, etc., are confused with typhus fever, to identify absolutely the nature of the virus. To this end, the virus from patients was transmitted to guinea-pigs. Previous observers have demonstrated that such virus circulating in the blood during the febrile period, when inoculated into guinea-pigs (or monkeys), produces a febrile reaction of from 4 to 11 days' duration after an incubation period of usually from 7 to 14 days (Ricketts and Wilder\*). The guinea-pig's blood during the febrile period is infective for other guinea-pigs.

In the following series of experiments (see Table 1) we have demonstrated that the virus with which we were dealing was typical of typhus fever. The usual procedure was to inject into guinea-pigs

\* Anderson (Jour. Med. Research, 1914, 30, p. 467) in an exhaustive study finds the incubation period among guinea-pigs to be from 4 to 29 days; in most cases between 7 and 10 days, and in 91% of cases between 7 and 14 days. The duration of illness is from 4 to 18 days; in 80% of cases it is between 7 and 11 days.

<sup>2</sup> Jour. Infect. Dis., 1915, 17, p. 1.

intraperitoneally 3 c.c. of the patient's blood (defibrinated) plus 3 c.c. of normal salt solution. After an average incubation period of 12 days the guinea-pigs would develop fever. Such animals were bled and the virus transmitted to others. The virus was maintained for 2 generations, the work being then interrupted. The period of incubation, the type of the febrile reaction, and the findings at autopsy — enlarged spleen with prominent Malpighian bodies (first noted by Baehr) and absence of other gross lesion — corresponded in every way with the results obtained in previous work with the virus of endemic and epidemic typhus fever.

TABLE 1  
VIRUS TRANSMISSION EXPERIMENTS

Patient	Incubation Period in Guinea-pig, Days	Febrile Period	Autopsy	Transmission
1	14	Died after 5 days	Spleen slightly enlarged; prominent Malpighian bodies. No other lesion	None
6	11	Bled after 2 days	Same.....	After 10 days guinea-pig developed fever. Re-transmitted
14	12	Died after 1 day	Same.....	None
16	12	Bled after 3 days	Same.....	Transmitted, but guinea-pig died in 2 days, no cause being found

Several guinea-pigs injected with the blood of patients not suffering from typhus fever failed to show the reactions described.

#### RESULTS OF BLOOD CULTURES

Blood cultures were taken on all the available cases of typhus fever and on numerous controls. It is important to note that the absence of hospital facilities and nursing, and the necessity of working in the filthy houses of ignorant natives, forced an adaptation of the technic to the surroundings and may have interfered somewhat with our results.

Blood cultures were taken on 31 cases of typhus fever as well as on several patients not having this disease. With the exception to be mentioned, the technic and the culture media employed were those used in the previous studies by Plotz.<sup>2</sup>

Briefly, the technic was as follows: After preliminary sterilization, 12 to 20 c.c. of blood were aspirated from a vein in the antecubital space. Two cubic centimeters were inoculated into each of 6 to 9 tubes. These tubes, measuring 15 cm. in length and 2 cm. in diameter, contained 20 c.c. of 2% glucose agar (+0.9 reaction), to which 4 c.c. of ascitic fluid were added. We wish to emphasize at this point that the ascitic fluid used was tested previously both aerobically and anaerobically, that it was bile-free, and had a specific gravity of at least 1.015. The blood and nutrient media were carefully poured from one sterile tube into another, so that thorough mixing took place. The contents were then allowed to solidify in an upright position, the surface was layered over with melted agar, and the whole then incubated at 37 C.

Of the 31 blood cultures made on typhus-fever patients, 3 were taken with 0.5% glucose serum agar. Of these three 1 was positive (see Table 2). Of the 28 cultures made with 2% glucose serum agar, only 8 could be followed a sufficiently long time, unsettled political conditions forcing our departure before the remaining cultures could be satisfactorily studied. All of these eight showed an organism the identity of which with Plotz's bacillus—*B. typhi-exanthematici*—will be discussed later.

TABLE 2  
RESULTS OF BLOOD CULTURES

Case	Time of the Taking of Blood Cultures		Amount of Blood Cultured, c.c.	Number of Colonies	Day of Appearance of Colonies in Culture	Media
	Days Before Crisis	Day of Disease				
1	5	6th	10	1 1 8	7th 12th 14th	0.5% glucose serum agar
3	At crisis	At crisis	12	Negative		
9	At crisis	At crisis	15	Negative		
12	5	9th	8	2	9th	2% glucose serum agar
13	8	6th	8	3 1	7th 9th	
14	6	9th	16	3 2	5th 7th	
15	3	8th	16	2	6th	
16	3	6th	2	1	9th	
21	5	5th	12	1	9th	
22	Died	4th	9	3	6th	
40	Unknown	3rd	12	6	4th	

As Table 2 shows, the two cases in which there were no organisms were cultured at the crisis. This fact, as well as the greater number of colonies appearing in those cultured up to the 6th day of the disease, conforms with findings made in the original studies by Plotz, Olitsky, and Baehr. They found that blood cultures on patients suffering from the disease showed a larger number of colonies on the 4th and 5th days before the crisis than on the few days immediately preceding the

crisis, and that those on guinea-pigs inoculated with typhus virus showed the largest number on the 2nd and 3rd days of illness. In either case blood cultures were rarely positive at the time of the crisis.

The total number of colonies, 34, as compared with the total number of cubic centimeters of blood cultured, 121, shows the interesting relation of the degree of bacteriemia to the severity of the cases. Comparing the results obtained in Mexico with those obtained in New York (in studies made on endemic typhus fever as well as on the epidemic type in Serbian immigrants) we note the following:

TABLE 3  
COMPARISON OF DATA SHOWING RELATIONSHIP BETWEEN BACTERIEMIA AND SEVERITY OF DISEASE IN TYPHUS FEVER

Type of Disease	Mortality	Cubic Centimeters of Blood Cultured	Number of Colonies	Average Number of Colonies per c.c.
Endemic typhus (New York).....	About 0.3%	436	33	0.07
Mexican typhus.....	20%	120	34	0.28
Epidemic (Balkan, 1914).....	18 - 60%*	51	74	1.45

\* Eighteen percent represents the mortality in our small series of cases (11) studied in New York. Reports from the Balkans place the average percentage at much higher figures. In a report based on studies of typhus fever in Bulgaria and Russia in 1915-1916 by Plotz and Baehr, which will appear shortly, the results of blood cultures correspond with those given here; the epidemic was midway, as regards severity, between the endemic and the Mexican types, the blood cultures showing more positives than in the endemic, and fewer positives than in the Mexican cases.

Thus we see that the more severe the disease, the greater is the number of colonies. This is exemplified also in our series in Case 1. This patient was most desperately ill and was in extremis at the time of the crisis, but recovered after a very stormy convalescence. Our fatal case likewise had more than the average number of colonies.

The day of appearance of these colonies in the original blood cultures fell between the 4th and the 14th days, usually about the 9th day. Since these colonies are first seen only after they have attained considerable size (the opacity of the culture media interfering with their detection), it is logical to assume that the colony grows very slowly at first, requiring a considerable period of time before reaching a visible size.

Blood cultures were also taken at the same time in a number of patients suffering from fevers other than typhus fever, such as influenza, bronchitis, bronchopneumonia, pneumonia, cystitis, etc. All were taken during the febrile period of the disease, the same serum and media and technic being used as for the typhus cases. The total

number of cubic centimeters of such blood cultured was 76, as against 120 of typhus-fever blood. The blood cultures in control cases were observed a sufficiently long time, usually for about 21 days. All these cultures were either negative or contained the exciting cause of the disease, as influenza bacilli, pneumococci, etc.

#### RESULTS OF AGGLUTINATION TESTS

In studying the serologic reactions found in cases of Mexican typhus fever, we were forced, because of a lack of facilities and animals, to place our main reliance upon agglutination tests. The serum obtained from patients was usually small in amount, hence the microscopic method was used. Wherever the quantity of serum was sufficient, however, macroscopic tests were employed as well, the result usually confirming the microscopic.

The method of setting up these tests was as follows:

The blood was collected in capillary tubes and the serum allowed to separate by standing or by centrifugation; it was then heated to 56 C. for one-half hour. Dilutions of the inactivated serum of 1:25, 1:50, 1:100, and 1:200 were made in small sterile tubes, so that by the addition of equal quantities of agglutinin these dilutions were doubled; that is, they were from 1:50 to 1:400. The agglutinin was prepared from growths of the organism on agar slants, a sufficient amount being washed off into 0.9% salt solution to make a suspension of proper opacity. This was shaken and then centrifugated gently so as to throw down any clumped masses of bacteria. The tests were set up in hollow ground slides, kept at room temperature for exactly 1 hour, and then read. In reading, the usual caution was taken to avoid the natural clumping incident to drying or that which occurs about the very edge of the material. Proper controls were always employed. When macroscopic tests were made, 1 c.c. of each of the dilutions of the inactivated sera was added to 1 c.c. of the bacterial suspension. Such tests, put up in sterile tubes, were incubated 1 hour, then kept at room temperature 24 hours, and afterward read.

In regard to the strains used in the agglutinin, we found, on several occasions, a freshly recovered organism to be inagglutinable, even in the serum of the patient from which the organism was obtained. However, such serum would agglutinate actively older strains—for instance, Strain 10, isolated about 2 years previously from an epidemic case. This experience corresponds with that encountered at times with the typhoid bacillus.

Again, as a consequence of the lack of hospital facilities, most of our patients could be bled only once, either before or after the crisis, and we were able, therefore, to test for the presence of agglutinins at but one period of the disease. In a few, however, we could trace the

TABLE 4  
RESULTS OF AGGLUTINATION TESTS AT DIFFERENT STAGES OF THE DISEASE

Case	Days before Crisis	Result	Critical	Days after Crisis	Result
1	...	.....	.....	5	1:50 +
3	...	.....	.....	9	1:100 +
6	...	.....	.....	13	1:200 +
7	...	.....	.....	3	1:600 +
9	...	.....	.....	16	1:200 ++
11	4	1:200 +	1:200 +	19	1:100 ++
12	1	1:50 —	.....	16	1:400 +
13	...	.....	.....	4	1:400 +
14	...	.....	.....	4	1:800 +
15	3	1:100 +	.....	6	1:400 +
18	...	.....	.....	..	.....
19	...	.....	.....	8	1:50 +
20	...	.....	.....	7	1:800 +
21	...	.....	.....	2	1:400 +
23	...	.....	.....	9	1:50 —
25	5	1:50 —	1:50 +	8	1:200 +
27	8	1:50 —	.....	..	.....
28	6	1:50 —	.....	..	.....
29	2	1:50 —	.....	6	1:100 +
30	1	1:50 —	.....	8	1:800 +
31	...	.....	1:200 +	7	1:200 ++
32	6	1:50 —	.....	..	.....
34	6	1:50 —	.....	..	.....
36	...	.....	1:50 +	..	.....
37	8	1:50 —	.....	..	.....
38	6	1:50 +	.....	..	.....
39	10	1:50 —	.....	..	.....
40	10	1:50 —	.....	..	.....
41	8	1:50 —	.....	..	.....
42	2	1:50 —	.....	..	.....
43	9	1:50 —	.....	..	.....
44	11	1:50 —	.....	..	.....
46	...	.....	.....	?	1:800 +

development of these antibodies during the various stages of the disease.

The total number of tests made was 41; 18 being made at the height of the disease, 4 at the crisis, and 19 after the crisis. There were 16% positive results in the antecritical period. In all these the agglutination showed a low titer. Of 4 tests made on bloods taken at the crisis, all were positive, but here as well the agglutination limits were low (from 1:50 to 1:200 positive). Of the tests made after the crisis, all but 1 were positive, giving a percentage of positives of 95. Also, the agglutinin titer of the serum showed much stronger reactions, several agglutinations occurring in dilutions of 1:800.

These results correspond for the most part with those obtained in the study of endemic and epidemic typhus fever in New York. That is, the agglutinins in typhus fever are usually negative at the height of the disease but increase in quantity as the crisis is reached and become almost uniformly positive in the postcritical period.

At the same time control agglutination tests made with the same technic were set up with serum obtained from patients suffering from diseases other than typhus. In this group were included febrile cases in which the tests were made not only at the height of the fever, but also in the postcritical stage. In most of these cases, the tests were negative; in one there was agglutination in a dilution of serum of 1:50. However, it has been emphasized previously that reactions in dilutions of serum up to 1:50 may not be specific. This occurs as well with other organisms.

We shall state later the results obtained in agglutination tests with typhus immune serum against agglutinogens of other than typhus organisms.

#### IDENTIFICATION OF THE ORGANISM

The identification of the organism recovered from the blood of Mexican typhus-fever patients was based upon its definite characteristics. These refer to the appearance of the colony, the morphology of the bacillus, the cultural characteristics including fermentation reactions, and finally its behavior towards typhus immune serum, especially in regard to agglutination and complement-fixation. In all these respects the organisms we have cultured from typhus in Mexico, including the organisms obtained from cultures from lice and from experimental typhus fever in guinea-pigs, correspond with *B. typhi-exanthematici*.

The characteristics of the organisms isolated are as follows:

*Colonies.*—The colony as it appears in blood cultures usually attains its maximal size by the 9th day, in one case in our series (see Table 2) as early as the 4th day. At this time, the colony appears as a small (3 to 4 mm.) opaque spot surrounded by a well-defined brownish area of precipitation. On cross section it is brownish in color, and if allowed to develop in the culture tube for a time, it becomes Y-shaped, the arms of the Y being whitish and fusiform.

*Morphology.*—The organism is a small slender bacillus averaging about a micron in length. When very young, it appears to have a morphology similar to the small-type influenza bacillus. It is usually gram-positive, altho we have met with many strains, especially from louse cultures, which were decolorized by Gram's method. This is true in the early subcultures; in the later subcultures the predominating organisms are gram-positive, while numerous gram-negative bacilli may be seen scattered throughout the field. We have not been able to determine polar bodies in young cultures, but in older cultures they are apt to appear occasionally, along with degeneration and involution forms.

*Cultural Characteristics and Fermentation Reactions.*—Culturally the Mexican strains are identical with the organism isolated in New York. It is a strict anaerobe. It grows rather sparsely on 0.5-2% glucose serum agar slants, so

that it is necessary to transplant a considerable amount in order to obtain sufficient growth. This medium shows a growth in 3 days after inoculation and is precipitated more or less, depending on the age of the culture. In these and other respects it corresponds with the description detailed in the work previously cited.<sup>2</sup>

It is important to emphasize in regard to fermentation reactions that the purity of the carbohydrate used in the media for the tests is of greatest significance in obtaining uniform results. In the reactions to be described, for example, the observations were made on media prepared with crystallized lactose; media prepared with powdered lactose gave marked variations, possibly due to impurities in the latter.

In order to ascertain the reliability of the carbohydrates used, it is best to note previously the reactions of a standard strain of a known organism, for example, the typhoid bacillus, upon the same sugars. Again, as suggested by Dr. Libman, one may find it necessary to purify a sugar, as in the case of lactose containing traces of dextrose and galactose, by subjecting the carbohydrate to the growth of an organism which will use up the undesirable elements, this measure being based on the method of Theobald Smith for eliminating dextrose from meat infusions by inoculation with *B. coli*. In the case mentioned, lactose, the paratyphoid strains will ferment out the impurities and leave the lactose untouched. Of course, it becomes essential to test the resultant sugar for its efficacy, and this may be done by growing in it a known organism, as *B. coli* in the case of lactose. The *modus operandi* should also allow no extraneous factor to complicate the results. The pyrogallie acid used in the production of anaerobiosis, if impure, may liberate volatile acids which cloud the readings.

Our methods of procedure were as follows: The medium was made of 3% agar, containing 1% of the carbohydrate to be studied, to which were added one-third as much of rich ascitic fluid, and a sufficient amount of litmus (Kahlbaum) to color the entire material a deep blue. Just before use reactions of such media were usually 0.8% acid. Slants made this way were inoculated with a quantity of the culture and were allowed to incubate for 6 days. For suitable anaerobic conditions we found that the use of jars subjected to a negative pressure of 600 mm. Hg, which at the same time had a compartment for the pyrogallie acid with the potassium hydrate, were more suitable than Buchner tubes. All the tubes were influenced, in the former case, by the very same conditions, and the extraneous acid was less likely to complicate the results.

TABLE 5

RESULTS OF FERMENTATION TESTS, "A" SIGNIFYING ACID-PRODUCTION, AND "P" PRECIPITATION

Carbohydrate	Epidemic N. Y.	Endemic N. Y.	Mexican
Lactose.....	Negative	Negative	Negative
Glucose.....	A, P	A, P	A, P
Raffinose.....	Negative	Negative	Negative
Maltose.....	A, P	A, P	A, P
Mannite.....	Negative	Negative	Negative
Galactose.....	A, P	A, P	A, P
Inulin.....	A, P	A, P	A, P
Saccharose.....	Negative	Negative	Negative
Dextrin.....	Negative	Negative	Negative

The Mexican strains, tested in this manner, gave the following reactions: acid-production (but no gas-formation) on glucose, maltose, galactose, and inulin; no acid (nor gas-production) on lactose, raffinose, mannite, saccharose and dextrin. A comparison of these with the results obtained in New York for epidemic and endemic strains of the typhus organism is shown in Table 5.

Incidentally, we may state that growth was luxuriant on the inulin, maltose, and glucose media, but sparse on the lactose, mannite, and saccharose media.

Thus we see the complete correspondence, as regards the reactions on the various carbohydrates, of the Mexican strains of the typhus bacillus with those of the epidemic and endemic organisms isolated in New York.

*Serum Reactions.*—The most important methods for the identification of the organisms obtained from Mexican typhus fever are based on the immune-serum reactions. For the purpose of such study we depended on the use of human serum obtained from typhus immune subjects. In order to make comparative studies on an organism grown on human serum media, as in this instance, it is of prime importance to use human immune serum, since the use of artificially prepared immune serum, as that produced in rabbits by the repeated injection of the organism, may give results which are nonspecific. One of us (Olitsky) in collaboration with Bernstein<sup>3</sup> has found that artificially prepared immune serum, as in rabbits, against organisms grown on serum media gives results which are nonspecific. This is due to the fact that such immune serum contains, besides the specific antibodies, antibodies developed against the serum (or protein) employed in the media upon which the bacteria in the antigen were grown. The serum antibodies give rise to nonspecific agglutinins, precipitins, complement-fixing bodies, and cellular antibodies.

In the following tests, we used endemic-typhus immune serum, as this type was the only one available.

(a) *Agglutination Tests.* For these studies there were available 5 strains of the Mexican organisms—No. 204, from a louse culture; No. 207, from the spleen of a guinea-pig infected with typhus virus; No. 208, from a human-blood culture in a case of typhus fever; Nos. 209 and 212, from cultures of lice.

The methods employed were the microscopic and macroscopic. The agglutinogens were prepared from each of these strains in the manner stated. The immune serum was inactivated and diluted so that the final dilutions were 1:50, 1:100, 1:200, and 1:400.

<sup>3</sup> Jour. Infect. Dis., 1916, 19, p. 253.

TABLE 6  
AGGLUTINATION TESTS: ENDEMIC-TYPHUS IMMUNE SERUM AGAINST MEXICAN STRAINS

Strain	Control	1:50	1:100	1:200	1:400
204.....	0	++	++	++	+
207.....	0	0	0	0	0
208.....	0	++	++	+	0
209.....	0	++	++	++	+
212.....	0	++	++	+	0
Nontyphus organisms...	0	0	0	0	0

By reference to Table 6, we find that of the 5 strains studied, 4 gave positive agglutination reactions with the endemic-typhus immune serum. One may explain the nonagglutinability of the remaining strain—which, however, bound complement—by the fact that endemic-typhus serum was used. It has been shown previously that various strains differ in their serum reactions and that polyvalency in such tests is of considerable importance. The same serum was found to agglutinate New York strains of the organisms strongly in dilutions of 1:400. Hence, as far as these tests go, one may conclude that the Mexican organisms are strains of the typhus bacillus.

Incidentally, the typhus immune serum was tested against agglutinogens made of organisms resembling somewhat the typhus bacilli. These bacteria were all gram-positive, but differed in one or more of the characteristics mentioned, from the typhus bacillus. Some grew aerobically after a few subcultures; others, while strictly anaerobic, differed in morphology and growth. One of these organisms was a typical *B. acne*; two others were isolated from pustules in cases of acne vulgaris; one was obtained from a blood culture from a patient suffering from chronic polyarthritis, and one from a blood culture in a case of chorea. However, when these bacteria were tested against the typhus immune serum, the same technic being used as indicated heretofore, the results were uniformly negative, no agglutinations occurring even in very low dilutions of the serum.

(b) Complement-Fixation Tests. All the available strains isolated from cases of Mexican typhus fever were studied by means of complement-fixation tests to determine their relationship to those obtained in New York.

For this purpose individual antigens were prepared from each strain. The methods of preparation were two. In the first instance the growth of the organism was suspended in distilled water and heated at 60 C. for 1 hour, subsequently being autolyzed at 37 C. for 24 hours. In the second case the

procedure was continued to include filtration through a Berkefeld candle (Size N— pressure 100 mm. Hg), and heating at 56 C. for one-half hour on each of 3 consecutive days. The second method is preferable, producing a lasting, nonanticomplementary "antigenic," antigen; the first commends itself only for the rapidity of its preparation. Never more than one-fourth the anticomplementary unit was employed.

The serum used was obtained from a patient who had recovered from endemic typhus fever. It was inactivated at 56 C. for one-half hour and was absolutely nonanticomplementary.

The hemolytic system consisted of antishoop rabbit amboceptor (titration varying from 1:1000 to 1:10,000), a 5% suspension of sheep cells, and guinea-pig serum (1:10) as complement. The quantities employed were one-half the amounts used in the original Wassermann reaction. The system worked perfectly for the performance of routine Wassermann tests. The possible presence of natural amboceptor was always considered.

The time allowed for fixation was one-half hour at 37 C. in a water bath, then 3½ hours in the icebox.

The individual antigens were made up of the strains mentioned (see "Agglutination Tests").

Altho no attempt was made to study quantitatively the amount of complement-fixing body by using higher dilutions of the immune serum, yet the correspondence of the results presented in Table 7 shows conclusively that the Mexican organisms are similar to those isolated in New York in cases of endemic and epidemic typhus fever.

At the same time comparative studies were made with organisms having some of the characteristics of the typhus bacillus. The source of these organisms was mentioned previously under "Agglutination Tests," where it was stated that as agglutinogens they failed to give agglutination with typhus immune serum. Table 8 shows that typhus immune serum in the presence of such antigens bound no complement and that this serum did bind complement in the presence of typhus-bacillus antigen. In these tests all antigens, as well as the immune serum, completely hemolyzed by themselves in the control tubes. Altho the nontyphus antigens were used, as indicated in Table 8, in amounts nearer the anticomplementary unit and in generally greater quantities, the results were uniformly negative.

These tests in conjunction with the agglutination reactions formed the most reliable methods of differentiation of the Mexican strains from organisms resembling them in one way or another. An important factor, however, should not be overlooked, namely, that comparative studies should always be made with an immune serum from the same species from which the antigen to be investigated is drawn. Otherwise nonspecific results may be expected.

TABLE 7  
COMPLEMENT-FIXATION TESTS WITH MEXICAN STRAINS

Strain in Antigen	Endemic-Typhus Immune Serum		Normal Human Serum	
	0.05	0.10	0.05	0.10
204.....	++++	++++	—	—
207.....	++++	++++	—	—
208.....	++++	++++	—	—
209.....	++++	++++	—	—
212.....	++++	++++	—	—
Polyvalent N. Y. typhus antigen....	++++	++++	—	—
Nontyphus antigen.....	—	—	—	—

TABLE 8  
COMPLEMENT-FIXATION TESTS WITH NONTYPHUS ANTIGENS

Source of Antigen	Dosage of Antigen, c.c.	Endemic-Typhus Immune Serum		Normal Human Serum	
		0.05 c.c.	0.10 c.c.	0.05 c.c.	0.10 c.c.
B. acné.....	0.3	—	—	—	—
Gram-positive bacillus from an acne case.....	0.2	—	—	—	—
Gram-positive bacillus from another acne case..	0.3	—	—	—	—
Gram-positive bacillus from blood culture in chorea (strict anaerobe).....	0.2	—	—	—	—
Gram-positive bacillus from blood culture in chronic infectious arthritis (strict anaerobe)..	0.3	—	—	—	—
Polyvalent typhus bacilli.....	0.15	++++	++++	—	—

#### PATHOGENICITY OF THE ORGANISM

Previous experiments by Plotz, Olitsky, and Baehr<sup>2</sup> demonstrated that organisms obtained from epidemic typhus fever are pathogenic for guinea-pigs only when inoculated from early subcultures. These studies showed also that the organisms isolated from endemic cases fail to give such reactions. The conclusion was reached that there is a marked loss of virulence of the bacilli after transplantation on slants of 2% glucose ascitic agar. With this in mind, we attempted to inoculate into animals the original colony of the organism (as it occurs in the blood culture) and we obtained thereby very striking results.

Guinea-pig B1 was injected on Feb. 12, 1916, with 1 loopful of a colony growth from a blood culture (Case 1—the colony having appeared 7 days after the taking of the blood culture). The growth was emulsified in 2.5 c.c. of salt solution and injected intraperitoneally. There was a rise in temperature

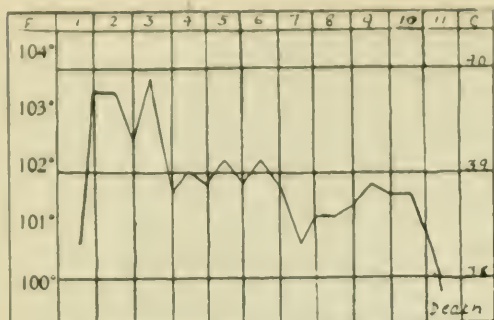


Chart 1. Temperature record of Guinea-pig B1, injected with organisms directly from colony in blood culture.

for 48 hours after the injection. The animal then became gradually feebler and died on February 23, 11 days after inoculation (see Chart 1). The injection of typhus virus into guinea-pigs is followed occasionally by the same reaction. Autopsy showed what we consider typical of typhus fever in the guinea-pig. There was no peritonitis, nor any other lesion except the slight enlargement of the spleen with hypertrophy of the Malpighian bodies. At the same time the spleen was removed in sterile manner, macerated in salt solution, and cultured according to the usual technic. Six days

later 3 colonies appeared containing the typical typhus bacillus.

To summarize, the main points of this experiment are: first, the initial rise of temperature corresponding with similar rises obtained with epidemic strains and not with endemic, due very probably to the endotoxins of the organism; second, death after a period corresponding with the average incubation period of typhus fever in guinea-pigs; third, the typical lesions of typhus at postmortem examination; and last, the recovery of the organism from the spleen culture. In other words, the injection of the organism caused typhus fever in this guinea-pig.

Guinea-pig B2 was injected intraperitoneally with 1 loopful of the agar emulsified in 2.5 c.c. salt solution, of the same blood culture, as a control on Guinea-pig B1. This was done to eliminate from the experiment the factor that the infective agent may have been carried over from the blood in the medium. This animal showed no such reaction.

To study the influence on virulence of growth on the media ordinarily used for culture, the following experiments were performed:

Guinea-pig C1 was injected with a 3-day growth on a 0.5% glucose serum agar slant of the identical organisms found in the foregoing colony—that is, the first transplant from the blood culture of Case 1. The entire growth on one standard slant was emulsified in 3 c.c. of salt solution and injected intraperitoneally. There was no preliminary rise in temperature as was seen in Guinea-pig B1 (which we have ascribed to endotoxin action). However, after 12 days the animal suddenly died, before we could make our blood cultures.

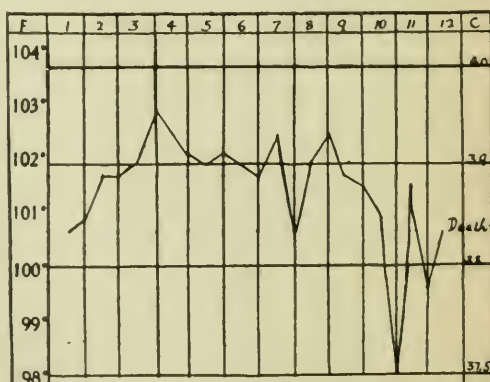


Chart 2. Temperature record of Guinea-pig C1, injected with the first transplant.

This death was preceded, the day before, by a marked drop in temperature (see Chart 2). The autopsy showed no lesions except in the spleen, which was not enlarged, but was slightly congested, with Malpighian bodies prominent. To summarize, there was no toxic action (showing itself in an early rise of temperature), the incubation period was typical, and the postmortem observations corresponded with those of the lesions in typhus fever in guinea-pigs. In other words, there was presumptive evidence of typhus fever as having been produced by the injection of the first transplant of the organism.

The effect of a retransplantation is shown as follows: Guinea-pig C5 was injected intraperitoneally with the second transplant of the very organism occurring in the colony just mentioned (from Case 1). The entire growth for 4 days on a slant of 0.5% glucose serum agar was emulsified in 3 c.c. of salt solution and inoculated. There was no rise in temperature, either early or after the usual incubation period. Here, then, we have absolute loss of virulence (see Chart 3).

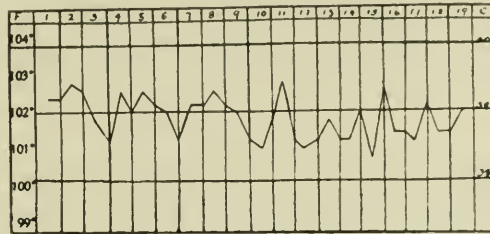


Chart 3. Temperature record of Guinea-pig C5, injected with second transplant. This animal was alive and well after 19 days' observation, work having been interrupted at the end of this time.

In agreement with previous observations we may conclude that this organism is pathogenic only in its very youngest generation and that it loses its virulence almost immediately on being subcultured on the glucose serum media.

#### ANIMAL EXPERIMENTS WITH INFECTED LICE

It was our purpose to produce typhus fever in guinea-pigs by the intraperitoneal injection of infected lice so that the nature of the reaction could be studied bacteriologically.

Previous workers, notably Anderson and Goldberger, Nicolle and Conseil, Ricketts and Wilder, and others, have been able to infect monkeys by the injection of such body lice subcutaneously. In these instances the number of lice varied from 37 to over 100. Usually no fever or other manifestations of the disease were produced, except the development of an immunity to a subsequent inoculation of typhus fever virus. Direct transmission of the disease to guinea-pigs, with the typical rise in temperature, by means of infected lice has not thus far been attempted.

With this end in view, we collected from the clothing or skin of the patients ill with typhus fever, the lice which were used in the following experiments. That these lice either had fed recently or were still feeding actively when removed from the patient, was indicated by their distention with blood. No attempt, however, was made to start with normal lice and allow them to feed on patients in a definite manner. Interruption of the work precluded this. The lice were picked off the clothes or patient with forceps, gathered in a Stender jar containing a piece of sterilized cheese-cloth, and transplanted to the laboratory where they were immediately injected.

They were prepared for injection as follows: Into a sterile mortar were placed 4 c.c. of salt solution, the desired number of lice were put into this salt solution, and with the aid of forceps the lice were crushed thoroughly with the pestle until a homogeneous emulsion resulted. This was then injected intraperitoneally into guinea-pigs. No effort was made to study the infectivity of the separate parts of the louse (salivary glands, intestinal contents, etc.).

Guinea-pig C6 was injected with the emulsion of 30 lice obtained from Case 18 when this patient was in the 13th day of illness, or 1 day before his crisis. Since it has been previously determined that lice become infectious after 5 days from the time of their feeding on infected blood, it was presumed that they carried the infectious agent at that time. On the 9th day after injection the animal experienced a sudden drop in temperature followed by a rise to 104.4 F. (see Chart 4). For further study the animal was exsanguinated and its defibrinated blood injected into 3 other guinea-pigs. The work was then interrupted and the transmission of the virus could not be followed. However, autopsy on the original guinea-pig showed no lesion except in the spleen, which was enlarged and congested with its Malpighian bodies prominent — typical of typhus fever in guinea-pigs.

The spleen of this animal was removed in a sterile manner. It was macerated in sterile salt solution and the emulsion thus obtained was cultured in deep tubes of 2% glucose serum agar. Five days later, 12 colonies of the typhus bacillus were obtained in one tube — the rest of the culture could not be observed longer.

A still more striking reaction was noted in Guinea-pig C8, which was injected in similar manner with 65 lice from Case 21. These lice were removed from the patient after the latter had passed the crisis (3 days postcritically). Unfortunately this animal could not be studied thoroughly, as the work was interrupted at about the time

of its reaction. But the definite incubation period of 10 days and the type of the rise in temperature lead us to believe that the guinea-pig had typhus fever (see Chart 5).

Thus we see that the transmission of typhus fever to guinea-pigs by the injection of infected lice has been accomplished and the same organism, the typhus bacillus, recovered from the organs of reacting animals.

Work was begun on the question of the bedbug as a carrier of this disease (along with the louse), but it was interrupted before satisfactory conclusions could be reached.

#### RESULTS OF CULTURES FROM INFECTED LICE

At the same time that the experiments just detailed were made, some of the lice were cultured.

Two methods were employed. In one instance the louse was seized between 2 sterile forceps, one grasping the thorax, the other the lower end of the abdomen, and the insect was pulled apart. The intestinal contents thus liberated were gathered in a platinum loop and inoculated into the media. In the second case the total number of lice were macerated in a mortar, with salt solution, and this suspension was cultured. The culture medium employed consisted of either a 2% or a 0.5% glucose serum agar. The tubes after inoculation were allowed to solidify in the upright position. We found these methods to be of value in separating the colonies of the organism from those of contaminating bacteria which are of course incidental to cultures of intestinal contents. Colonies, when present, usually appeared in the lower third of the tube.

Six series of experiments could be observed a sufficiently long time to note the final results. They are as follows:

Series 1.—Five lice, distended with the blood of Case 6, having been obtained from the patient 2 days before the crisis, or on the 12th day of the disease, comprised this series. They were cultured by one of the methods mentioned,

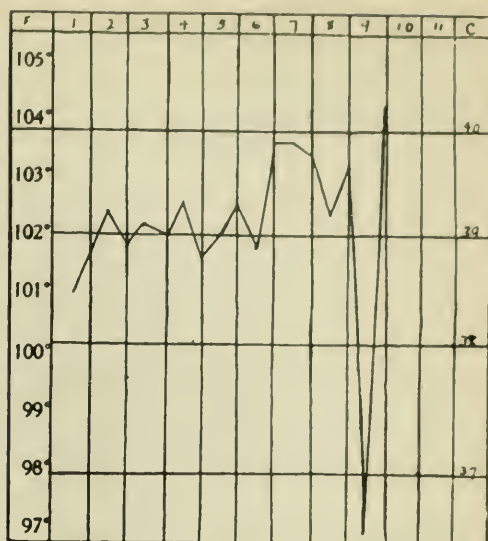


Chart 4. Temperature record of Guinea-pig C6, following injection of infected lice.

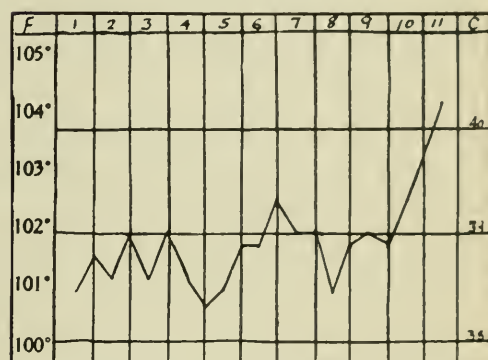


Chart 5. Temperature record of Guinea-pig C8, showing reaction after injection of 65 lice.

in 2% glucose serum agar in deep tubes. After 4 days of incubation very numerous minute colonies appeared. These colonies had the same characters as those of the typhus bacillus. They were apparently anaerobic, as they developed only in the deep portion of the tube, but it was impossible for us to subculture the organism. However, instead of being gram-positive, all these bacilli were uniformly gram-negative. We shall discuss later the significance of this variation in the staining properties.

Series 2.—Three lice taken from the same patient as Series 1, but in this instance 1 day before the crisis, or the 13th day of the disease, were cultured in deep tubes of 0.5% glucose serum agar. In one tube particular pains were taken to culture the excreta of a louse, passed on a piece of previously sterilized cheese-cloth. In this case, after 7 days' incubation, the most anaerobic portion of the culture medium was riddled with pinpoint colonies. These were made up of organisms having the typical morphology of the typhus bacillus, but, as noted, they were also gram-negative. Only one subculture (grown anaerobically) could be made and the organisms still retained their gram-negative character.

Series 4.—Two lice taken from the clothing of Case 12 at a period corresponding to 2 days before the crisis, or the 12th day of the disease, were cultured in 0.5% glucose serum agar in deep tubes. On the 11th day of incubation, 3 colonies appeared; on the 12th, 4. These colonies were of an organism which was subsequently studied in New York; it agreed absolutely in cultural and serum reactions with the typhus bacillus. In smears from the original colonies, most organisms were stained gram-negative, a few gram-positive; in later subcultures they were all gram-positive.

Series 6.—Six lice collected from the clothing of Case 13 by Dr. N. Frazin, were sent to us for culture. We are greatly indebted to Dr. Frazin, physician at Minas Dolores, not only for this courtesy but for numerous kindnesses shown us during the progress of the work. These lice were taken from the patient, who was suffering from a very severe form of the disease, at a period corresponding to the 12th day of the illness, or 2 days before the crisis. They were cultured in 2% glucose 3% agar serum media.

The culture of Louse 1 showed 1 colony after 3 days' incubation. The organisms were gram-positive. Cultures of Louse 2 showed 8 colonies after 3 days' incubation. All the organisms were gram-negative. Cultures from Louse 3 showed 4 colonies after 9 days' incubation. One colony was made up of gram-negative organisms, the others of gram-positive organisms. (A loopful of the growth in a colony made up of gram-positive organisms was emulsified in salt solution and injected into a guinea-pig. Our observations upon this animal, however, were unfortunately interrupted.) However, these organisms were subcultured and were brought back with us to New York, where they were compared with standard endemic and epidemic strains of the typhus bacillus and were found to be identical with them. Cultures from Louse 2 were gram-positive after first subculture; cultures from Louse 3 were gram-positive after second subculture.

Series 8.—Five lice taken from the clothing covering Case 18 at a period corresponding to 1 day before the crisis, comprised the series for culture, while 30 lice collected at the same time under the same circumstances were injected into a guinea-pig. This guinea-pig (C6) showed evidences of having been infected with typhus fever. The culture in 0.5% glucose serum agar was positive after 4 days of incubation. Here again the organisms were gram-negative in the original colony.

Series 9.—This series is of great importance inasmuch as the lice were collected from the body of Case 7, 4 days after the crisis. Four lice were cultured in deep tubes of 2% glucose serum agar. After 6 days' incubation the culture showed 2 typically growing colonies, one containing gram-negative organisms, the other partly gram-positive. After 2 subcultures these were uniformly gram-positive.

A loopful of organisms from an original colony were emulsified in salt solution and injected into Guinea-pig B9. Twenty-four hours later there was a drop in temperature of almost 4 degrees and on the 3rd day after injection the animal died. On autopsy the guinea-pig showed the typical lesions of typhus fever in this species: the spleen was slightly enlarged, and the Malpighian bodies were prominent. All the other viscera were apparently normal. The spleen was removed in sterile manner, macerated in salt solution and cultured. After 4 days' incubation 5 colonies appeared in one of the tubes, all organisms being gram-positive. The other culture tubes could not be observed long enough, the work being interrupted at about this time.

Several of the cultures of this series were studied in New York and were proved identical with the endemic and epidemic strains of the typhus bacillus.

To summarize the results of these louse cultures we may state that in practically all cultures that were made the organism was obtained. It was shown that lice contained the bacilli when they were taken from the patient at the height of the disease and 4 days after the crisis. This agrees with the findings of previous workers, who observed that a louse feeding on a typhus patient becomes infective for others after a period of 5 days. This period of time is necessary for the multiplication of the bacilli or their increase in virulence in the body of the louse until sufficient to make it infective. All the lice used in these experiments could be, then, infective.

The other striking observation was that these organisms are usually gram-negative in the original colonies. After one or two subcultures they become definitely gram-positive. The relationship between the virulence of the organisms and their staining reaction is very suggestive. Furthermore, it may be of interest to note that in previous work it was observed that in the original colonies in blood cultures from typhus cases the organisms were stained partly gram-negative and partly gram-positive in the same smear preparation. In recent studies by da Rocha-Lima<sup>4</sup> further corroboration of this fact is made; he finds that infective lice harbor an organism of similar morphology in enormous numbers, especially in the stomach wall, and remarkable to state, these organisms are gram-negative. Independently and at the same time Plotz and Baehr<sup>5</sup> in their studies on typhus fever in the Balkans and Russia, comparing normal with infected lice in smear preparations,

<sup>4</sup> Arch. f. Schiffs- u. Tropen-Hyg., 1916, 20, p. 17.

<sup>5</sup> Reports will appear in the Journal of Infectious Diseases.

found in lice from typhus patients numerous bacilli decolorized by Gram's method.

Again, we have demonstrated that in a guinea-pig injected with organisms from an original colony of a louse culture—that is, before they had lost their virulence by artificial cultivation—the bacilli caused the death of the animal in 3 days. The guinea-pig showed the typical findings, on autopsy, of typhus fever in this species, and from the spleen we have been able to cultivate the same organism.

Hence, in our studies on infected lice, we have been able to produce typhus fever by the injection of such lice, to recover the organism from the infected guinea-pig, to culture the organism from the typhus lice, and to demonstrate the pathogenicity of a culture of the typhus bacilli from the insect.

#### CONCLUSIONS

In the short period of our work (during which we received much assistance from Mr. Clarence A. Grabel, superintendent of the plant) we were able to confirm the results obtained previously by Plotz, Olitsky, and Baehr<sup>2</sup> and to add further evidence concerning the etiologic factor of typhus fever.

The data bearing on the etiologic relationship between this organism and Mexican typhus fever may be summarized as follows:

Of 11 cases cultured, 9 were positive. Of these eleven 3 were cultured in 0.5% glucose serum agar; 1 was positive. Eight of the eleven were cultured in 2% glucose serum agar; all these were positive. At the same time and by the same methods, blood cultures taken on control cases were negative.

The bacteria were more numerous in the early stages of the infection and in the more severe cases.

The organism obtained is identical in all its features, especially in carbohydrate-fermentation and in its serum reactions, with the typhus bacillus isolated in New York from cases of endemic and epidemic forms of the disease.

The blood so cultured was true typhus virus; we were able to infect guinea-pigs with typhus fever by the injection of such blood.

Further evidence to support our views was added by the nature of the formation of antibodies in the Mexican patients. We have been able to trace the development of agglutinins with these results: Before the crisis, 16% of the cases showed positive agglutinations; after the crisis, 95%. Agglutinins were absent in numerous control cases in

which a history of typhus fever could be excluded. Agglutinogens of various organisms, including those morphologically similar to the typhus bacillus, showed no agglutination with typhus-fever immune serum. Antigens made of these organisms likewise failed to bind complement in the presence of typhus immune serum.

Thus we see that specific agglutinins are present as a direct result of the typhus bacillus circulating at the height of this disease. These antibodies are usually absent when the fever is present, that is, when the antigen, the organism, may be recovered from the blood stream. After the crisis the agglutinins are evident in their greatest concentration.

We have been able to demonstrate the pathogenicity of the organism, and to show that the virulence deteriorates very rapidly in cultures. When an animal was infected in this way, the organism was recovered from its spleen.

In animal experiments with infected lice, we have been able to produce the disease in guinea-pigs when a sufficient number of lice were injected. From the spleen of one of these animals we obtained numerous colonies of the same bacillus.

Cultures from infected lice showed uniform results. The organism was recovered in all the cultures that could be observed the proper time, 21 days. The injection of an original colony into a guinea-pig caused the death of the animal in 3 days, and from its spleen the same organism could be isolated. The most striking features of these infected-lice cultures is the tendency of the organisms to be decolorized by Gram's method. This has occurred in every culture. The gram-negative bacilli, however, become gram-positive on subculture. It is logical to conclude from the evidence presented that the reaction of the organism to gram-stain has a distinct relationship to the maintenance and loss of its virulence.

Work on prophylactic vaccination will be reported in another communication.

## RELATION OF SPECIFIC PRECIPITATION TO OTHER IMMUNITY REACTIONS \*

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Within the last few years much interest has been manifested in the balance in the blood stream between the serum protease and its antiferment.

It has previously been supposed that, when protein antigens were injected into the body, specific proteolytic ferments were formed which would split the specific protein into simpler compounds. In the anaphylactic reaction a specific proteolytic ferment was thought to be formed which split the antigen on second injection, into toxic products. In the Abderhalden reaction the dialyzable substances were supposed to be formed by the action of a specific ferment on the substrate. The source of the split products was always believed to be the antigen.

It is easily shown that serum always contains a nonspecific protease that is normally prevented from acting by an antiprotease (antitrypsin). Serum will inhibit the proteolytic power of trypsin. The amounts of protease and antiferment seem to vary greatly under different conditions. Many have believed that the ferment-inhibiting substances of the blood are true antibodies, but very little proof has been advanced to support the view.

Jobling and Petersen<sup>1</sup> noticed that soaps of the unsaturated fatty acids inhibited the action of trypsin. From later experiments<sup>2</sup> they concluded that the ferment-inhibiting action of the serum is due to the presence of compounds of the unsaturated fatty acids. These same investigators showed<sup>3</sup> that sera from which the lipoids had been removed became toxic for the homologous animals. They explained the toxicity as due to exposure of the serum-proteins to the action of the serum protease with a consequent formation of toxic split products. They also observed changes in the coagulation of the blood showing that the mechanism of coagulation was altered. They believe therefore that the unsaturated lipoids play an important part in the mechanism of coagulation.

In anaphylactic shock there is no doubt that poisonous protein split products are the etiologic factors. This has been demonstrated in vitro. There is now a consensus of opinion regarding the rôle of protein poisons in the production of anaphylaxis, but there has been a diversity of opinion regarding the matrix, that is, the protein broken down.

Donati<sup>4</sup> concluded that the antigen was not split since organisms so used retained their antigenic properties. Now we find that so-called anaphylatoxin can be produced, in vitro, by treating sera with absorbing substances, such as kaolin, agar, starch, bacteria, etc.

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<sup>1</sup> Jour. Exper. Med., 1914, 19, p. 239.

<sup>2</sup> Ibid., p. 459.

<sup>3</sup> Ibid., p. 480.

<sup>4</sup> Arch. p. le. sc. med., 1912, 26, p. 421.

Jobling and Petersen showed lately<sup>5</sup> that serum antiferment (unsaturated lipoids) could be removed from sera by kaolin, starch, agar, and bacteria, and that sera treated in this way were toxic for the homologous animals. They concluded that anaphylatoxin represented serum rendered toxic by partial removal of the antiferment. They found that bacteria so treated were more resistant to the action of trypsin. From these experiments they placed the matrix of the protein split products on the serum proteins so exposed. They found that sublethal doses of soap solutions injected simultaneously with antigen prevented anaphylactic shock.

Recently Jobling, Petersen, and Eggstein<sup>6</sup> investigated the changes in serum ferments, antiferments, and protein split products during anaphylactic shock. They found that injection of the antigen into a nonsensitized animal was followed by practically no change in the serum ferments or protein split products. Further, that following the injection the body responded by a progressively increasing rapidity of mobilization of nonspecific protease, the amount of protease mobilized becoming greater as the maximum of sensitization was reached. Acute anaphylactic shock was accompanied by an immediate and marked increase in serum protease, by a fall in antiferment, by a rise in the aminoacid content and noncoagulable nitrogen in the serum, together with a primary decrease in serum proteoses. They summarized the mechanism of anaphylactic shock as: "An immediate mobilization of nonspecific protease in large amounts, the formation of antiferment deficiency through colloidal changes, a simultaneous cleavage of serum proteins through the peptone stage to amino acids, an intoxication by these peptones with a resulting cellular injury made evident by an increase in serum lipase, fall in temperature and other manifestations of acute shock. The elements of specificity lie in the mobilization of the nonspecific cellular ferment and the colloidal changes in the serum, not in the production of a specific ferment."

In the last few years much interest has been aroused in the Abderhalden reaction. Since this method has been available many investigators have published results in which the desired specificity was obtained, and others results which tended to discredit the specificity of the reaction. As stated before, it has always been assumed that the antigen itself is split by specific ferments. But specific ferments have never been demonstrated. The serum protease has been shown to be nonspecific. Jobling, Petersen, and Eggstein<sup>7</sup> have shown that the placental cells are not digested during the Abderhalden reaction, and that during the reaction the placental cells become more resistant to ferment action because of the absorption of serum antiferment. They concluded that the substrate digested is the serum proteins made available for protease action by the removal of the antiferment.

Bronfenbrenner<sup>8</sup> found that if placental cells were placed in contact with immune serum at 0 C. for 16 hours and then were removed, the serum was not digested, but that if the serum so treated was then incubated at 37 C., digestion took place. He found that placental cells previously treated with immune sera were capable of removing the antiferments from normal sera by contact at 0 C. Such normal sera when separated from these cells and incubated at 37 C. were digested. He removed normal serum from contact with this sensitized placenta and added fresh portions of normal serum to the same portion of treated placenta ten times, with the result that even the

<sup>5</sup> Jour. Exper. Med., 1914, 20, p. 37.

<sup>6</sup> Ibid., 1915, 22, p. 401.

<sup>7</sup> Ibid., 21, p. 339.

<sup>8</sup> Ibid., p. 221.

tenth portion of normal serum showed marked autodigestion when incubated at 37 C. This showed undoubtedly that the placenta once sensitized acquired the property of removing the antiferment from the serum. He also found that pregnant serum actually lost its specific substances during contact with placenta at 0 C. Returning the serum antiferment to the serum previously exhausted of its antiferment stopped the autodigestion. He concluded, "the Abderhalden test may be resolved into two phases: First, the sensitizing of the cells so that they attained the property of removing the antiferment, and second, the autodigestion of the serum." Smith<sup>9</sup> obtained similar results using bacterial cells instead of placenta.

It seems that in the Abderhalden reaction and the anaphylactic reaction the immune serum causes a change in the antigen, after which the antigen has attained the property of removing the antiferment from the serum. The antiferment being removed the nonspecific protease can digest the serum and thus form the protein split products.

It is known that the immune sera before the anaphylactic shock and the Abderhalden reaction contain precipitins. Also it has been noticed by Joachimoglu<sup>10</sup> that precipitins disappear instantly with anaphylactic shock. It is plausible to suppose that some change similar to a precipitin reaction must take place in these reactions. After observing these facts it occurred to me to ascertain to what extent the precipitate from a precipitin reaction would remove the antiferment from sera.

A rabbit was immunized to extract of fresh beef. After numerous injections the serum showed marked precipitation with beef extract. The rabbit was then bled and the serum obtained. The serum was added to ten times its volume of beef extract diluted 1:1000 and allowed to incubate for 2 hours. It was then placed in the ice box over night. The next day the precipitate was centrifugated from the supernatant liquid and washed four times. The precipitate, which measured 0.02 c.c. in a Hopkins tube after centrifugation for 10 minutes at high speed, was made up to 20 c.c. with normal salt solution. This suspension was used in the following experiments:

Volumes of 1 c.c. each of serum from a rabbit immunized to beef extract were mixed with varying amounts of suspension of precipitate. One tube was left as a control. These were all made up to the same volume and incubated for 3 hours. The total nitrogen and the noncoagulable nitrogen for each tube were determined.

The results of this experiment are shown in Table 1. The increase in noncoagulable nitrogen shows the amount of digestion. The suspension of precipitate contained only traces of noncoagulable nitrogen. The nitrogen was determined by the method of Folin and Denis<sup>11</sup> with slight variations. The coagulable and noncoagulable nitrogen were separated by adding to the tubes an equal volume of a

<sup>9</sup> Jour. Infect. Dis., 1916, 18, p. 14.

<sup>10</sup> Ztschr. f. Immunitäts., 1911, 8, p. 453.

<sup>11</sup> Jour. Biol. Chem., 1911-12, 11, p. 527.

solution of 10% glacial acetic acid and 20% sodium chlorid. The tubes were then boiled 5 or 10 minutes and the contents filtered through hard filter paper impregnated with kaolin.

TABLE 1  
RESULTS OF DIGESTION IN RABBIT IMMUNE SERUM AND SPECIFIC PRECIPITATE

Tube	Precipitin Rabbit Serum, c.c.	Suspension of Precipitate, c.c.	Total Nitrogen, mg.	Noncoagulable Nitrogen, mg.
1	1	0.8		0.31
2	1	0.4		0.35
3	1	0.2		0.43
4	1	0.1		0.36
5	1	0		0.07
6	1	0	2.01	

This experiment was now repeated numerous times with normal rabbit serum. Marked digestion was noted similar to that in immune serum. The results in one case are shown in Table 2. It was noted that a certain amount of the suspension is the most active; more than this amount or less does not produce as much digestion. This will be discussed later. From this experiment it seems that the precipitate will remove the antiferment from the normal serum.

TABLE 2  
RESULTS OF DIGESTION IN NORMAL RABBIT SERUM AND SPECIFIC PRECIPITATE

Tube	Normal Rabbit Serum, c.c.	Suspension of Precipitate, c.c.	Total Nitrogen, mg.	Noncoagulable Nitrogen, mg.
1	1	1		0.54
2	1	0.5		0.64
3	1	0.1		0.66
4	1	0.01		0.51
5	1	0		0.23
6	1	0	1.84	

Since Bronfenbrenner and Smith found that cells sensitized with their immune serum would remove the antiferment from all sera from the same species from which the immune serum had been obtained, and since the precipitate from a precipitin reaction removes the antiferment from the homologous normal serum, I tried to determine whether this reaction was specific for the homologous serum or not. I endeavored to find whether these substances would remove antiferment from other sera.

Colon bacilli were treated with serum from a rabbit immunized against *B. coli*. A 24-hour culture of *B. coli* was suspended in 3 c.c. of normal salt solution mixed with 3 c.c. of immune rabbit serum, and incubated for

2 hours. The organisms were then removed by centrifugation and washed four times. The bacilli, which now measured 0.05 c.c. in a Hopkins tube, were made up to 25 c.c. with normal salt solution. They were then used in the following experiments. The suspension contained only a trace of non-coagulable nitrogen. Experiments showed that in small quantities it caused digestion in normal rabbit serum. Jobling showed that normal bacteria in large amounts removed the antiferment from serum, but the removal would at least be small with such small quantities of normal bacteria.

Normal horse serum, sheep serum, and guinea-pig serum were treated with different amounts of sensitized colon bacilli and precipitate, both from rabbit serum. These were incubated 3 hours and then the total nitrogen and the noncoagulable nitrogen were determined, also the noncoagulable nitrogen in the controls. The results are shown in Tables 3, 4, and 5. It will be seen that there was a marked increase in the noncoagulable nitrogen in the sera so treated. All the experiments were repeated several times with similar results.

TABLE 3  
RESULTS OF DIGESTION IN NORMAL HORSE SERUM AND SENSITIZED COLON BACILLI

Tube	Normal Horse Serum, c.c.	Suspension of Precipitate, c.c.	Suspension of B. Coli, c.c.	Total Nitrogen, mg.	Noncoagulable Nitrogen in 1 c.c., mg.
1	3	0.5			0.60
2	3	0.1			0.62
3	3	0.01			0.58
4	3	0.001			0.46
5	3		1		0.56
6	3		0.5		0.58
7	3		0.1		0.62
8	3		0.01		0.65
9	3		0.001		0.39
10	1	0	0	2.12	0.40
11	1	0	0		

TABLE 4  
RESULTS OF DIGESTION IN SHEEP SERUM AND SENSITIZED COLON BACILLI

Tube	Sheep Serum, c.c.	Suspension of Precipitate, c.c.	Suspension of Sensitized B. Coli, c.c.	Total Nitrogen, mg.	Noncoagulable Nitrogen in 1 c.c., mg.
1	2	0.5			0.42
2	2	0.1			0.43
3	2	0.01			0.39
4	2		0.5		0.41
5	2		0.3		0.42
6	2		0.1		0.40
7	2		0.01		0.34
8	2		0.001		0.21
9	1	0	0	1.98	0.24
10	1	0	0		

TABLE 5

RESULTS OF DIGESTION IN GUINEA-PIG SERUM CAUSED BY PRECIPITATE AND COLON BACILLI FROM RABBIT SERUM

Tube	Guinea-Pig Serum, c.c.	Suspension of Precipitate, c.c.	Suspension of Sensitized B. Coll, c.c.	Total Nitrogen, mg.	Noncoagulable Nitrogen, mg.
1	1	1			0.50
2	1	0.1			0.52
3	1	0.01			0.49
4	1		1		0.49
5	1		0.3		0.51
6	1		0.01		0.34
7	1	0	0		0.41
8	1	0	0	2.02	

Since we should expect a similar and probably more pronounced action in pathologic sera, the serum of a tuberculous guinea-pig was treated in a similar way with sensitized colon bacilli. There was a marked increase in digestion, as shown in Table 6.

TABLE 6

RESULTS OF DIGESTION IN SERUM FROM A TUBERCULOUS GUINEA-PIG AND SENSITIZED COLON BACILLI

Tube	Tuberculous Guinea-Pig Serum, c.c.	Suspension of Sensitized B. Coli, c.c.	Total Nitrogen, mg.	Noncoagulable Nitrogen, mg.
1	1	1		0.55
2	1	0.1		0.56
3	1	0.01		0.43
4	1	0		0.33
5	1	0	2.08	

## DISCUSSION

These experiments show that the precipitate from a precipitin reaction will cause digestion in normal serum, and that this takes place with very small amounts of precipitate. Digestion is probably due to the removal of antiferments, allowing the nonspecific protease to act on the serum proteins. This seems to show the possible relationship of precipitins to other immunity reactions. Precipitins have been thought by many to be concerned in the anaphylactic reaction, some having thought precipitin and anaphylactin to be identical. Precipitin disappears after anaphylactic shock and passive anaphylaxis is conferred in proportion to the concentration of precipitin in the serum.

In the precipitin and anaphylactic reactions the serum has developed the property of reacting with the specific antigen with the result that a new body is formed in the serum. These experiments seem to show that this body now possesses the property of removing the antipro-

tease from the serum, or, rather, of rendering it inactive. Therefore, it seems possible that in anaphylactic shocks the serum proteins are exposed in this way to the action of nonspecific protease with toxic split products as the result. It seems that the mechanism of the Abderhalden reaction must be identical with the reaction mentioned. The immune serum has the same specific action on the surface of the cells, after which they possess the property of removing the antiprotease from the serum. After this removal the dialyzable peptones and amino-acids are formed by the action of the nonspecific protease on the serum proteins. Thus we see that the so-called precipitins may be concerned in the anaphylactic and Abderhalden reactions. In other words, it may be the precipitins which react with the antigens, thus forming new bodies which remove the antiprotease.

Bronfenbrenner has previously shown that the removal of antiprotease by sensitized cells is nonspecific in the homologous sera. My experiments seem to show that sensitized cells, and also precipitate formed by the action of immune serum and antigen, will not only remove the antiprotease from the homologous sera, but also from heterologous sera (horse, sheep, and guinea-pig). It therefore seems that this is a nonspecific reaction.

It is not clear just how the unsaturated lipoids are rendered inactive. One way might be by the saturation of the unsaturated bonds, but this is not likely. Again it may be by absorption of the lipoids, since the cells so treated become more resistant to the action of trypsin. This may take place with large amounts of kaolin, bacteria, etc., but it hardly explains the action of small amounts of precipitate and sensitized cells. A third method may be that of changing the state of dispersion of the lipoids. The unsaturated lipoids are inactive as ferment-inhibitors unless in a highly dispersed state. The action of precipitate and sensitized cells is probably that of changing the state of dispersion of the unsaturated lipoids so as to render them inactive.

It will be seen that the specificity in these reactions lies in the action of the immune serum on the antigen (a physical change). After this has taken place the removal of the protease-inhibitors (a physical change) and also the action of the serum protease (chemical change) are nonspecific.

It will be noticed that maximal digestion occurs with certain quantities of precipitate or sensitized organisms. Larger or smaller quan-

tities cause less digestion. Jobling called attention to the fact that there is a quantitative relation between the amount of kaolin and the maximal absorption of antiferment.

It will be noticed that sometimes with very small amounts of precipitate or sensitized organisms less digestion resulted than in the controls. Jobling also noticed similar results. It is suggested that in the case of bacteria this might be explained by the fact that the very small amounts may be dissolved and thus liberate the antiferment absorbed and also their own ferment inhibitors.

#### CONCLUSIONS

In small amounts the precipitate formed by action of immune serum and antigen, and also sensitized organisms, will remove antiferments from sera. This may show the relation of precipitins to anaphylactic and Abderhalden reactions.

The absorbing action of sensitized organisms and precipitate is not specific. They will remove the antiferment not only from the homologous sera, but also from heterologous sera.

There is a quantitative relation between the amounts of precipitate and sensitized organisms and the maximal amount of absorption of the antiferment.



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